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The involvement of the glucocorticoid receptor in regulating glucocorticoid ultradian rhythms

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The involvement of the glucocorticoid receptor in regulating glucocorticoid ultradian rhythms: the role of the FK506-binding protein 51

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Abstract

The hypothalamic-pituitary-adrenal (HPA) axis is a pivotal neuroendocrine system in mammals and the end product, glucocorticoids (GCs), are steroid hormones involved in several essential processes including cognitive functions, metabolic processes, and regulation of the inflammatory response. Glucocorticoids are secreted from the adrenal gland in response to stimulation by adrenocorticotropic hormone (ACTH), which in turn is secreted from the anterior pituitary gland following stimulation by corticotropin-releasing hormone (CRH).

The HPA axis is activated as part of the stress response and following circadian cues, and the overall GC secretion occurs in a circadian manner, with higher GC concentrations during the active phase (daytime in humans and nighttime in rodents). Moreover, this circadian variation is made up of high-frequency ultradian pulses, which have an important role in regulating gene expression in GC target tissues. Corticosterone (CORT) is the main GC in rodents, and studies in rats have shown approximately hourly pulses of CORT, which are preceded by pulses of ACTH.

Importantly, in humans, altered HPA axis activity is seen in various stress-related disorders including depression and anxiety.

Hypothalamic-pituitary-adrenal axis activity is regulated by a negative feedback loop that involves activation of the glucocorticoid receptor (GR). The GR, in turn, is regulated by its chaperone protein, FKBP51. Clinical studies have shown an association between FKBP51 polymorphism and the instances of depression. Thus, the general hypothesis is that increased FKBP51 expression can cause a hyperactive HPA axis and GC resistance.

To further investigate the role of FKBP51 in the regulation of HPA axis activity, two FKBP51-specific inhibitors were used to investigate their effects on the HPA axis in male and female rats. Serial automated blood sampling (ABS) procedures were performed in basal and stress-induced conditions following both acute, and sub-chronic (5-days) treatment with either a central and peripheral FKBP51-inhibitor (SAFit2), or a peripheral-only inhibitor (SAFit1). Furthermore, at what level of the HPA axis the SAFit compounds had their effects were investigated, by stimulating ACTH, and CORT secretion with CRH and ACTH, respectively, in SAFit2-, SAFit1-, or vehicle-treated rats. Moreover, the gene expression induction at 60

minutes post-noise stress in the hypothalamus and anterior pituitary gland following SAFit2, SAFit1, or vehicle administration were compared.

Acute treatment with SAFit2 in male rats resulted in lower maximum CORT, and lower stress-induced CORT compared to vehicle-treated rats. Similar results were observed following sub-chronic SAFit2 treatment, with lower mean, maximum, and basal CORT, and lower stress-induced CORT compared to vehicle-treated rats. Acute SAFit1 treatment had no effect in male rats, however shorter CORT pulses under basal conditions were observed, and a lower stress-induced CORT was observed following sub-chronic SAFit1 treatment in male rats compared to vehicle-treated rats.

In females, acute SAFit2 treatment had no effect, whilst acute SAFit1 treatment result in lower mean CORT, and lower stress-induced CORT compared to vehicle-treated rats. Sub-chronic treatment with SAFit2 in female rats caused lower mean, maximum, and basal CORT concentrations, but did not affect the stress-induced CORT.

Moreover, reduced CRH-induced ACTH secretion from the anterior pituitary gland was found in male rats treated for one day with either SAFit2 or SAFit1, suggesting a potential decrease in GR-mediated GC negative feedback.

These findings suggest a sexual dimorphism in the processes regulating GC-mediated negative feedback during basal conditions and following stress. Moreover, the sub-chronicSAFit1 treatment in male rats changes the pulsatility of ultradian pulses. This work has made important steps to further unravel the complexity of the HPA axis negative feedback and the sexual dimorphisms involved in this regulation.

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Author's declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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Table of Contents

List of tables and figures

List of abbreviations

2-AG	2-Arachidonoylglycerol
51KO	FKBP51 knock-out
ABS	Automated blood sampling
ACTH	Adrenocorticotropic hormone
AEA	Anandamide
AKT	Protein kinase B
AKT2	RAC-beta serine/threonine-protein kinase
ANXA1	Annexin 1
AP	Anteroposterior
AS160	Akt substrate of 160 kDa
AUC	Area under the curve
AVP	Arginine vasopressin
BAG1	BCL2 Associated Athanogene 1
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
BK	Large-conductance calcium- and voltage-activated potassium
BLA	Basolateral amygdala
BMAL	Brain and Muscle ARNT (Arylhydrocarbon Receptor Nuclear Translocator)-Like Protein
BNST	Bed nucleus of the stria terminalis
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CB1	Cannabinoid receptor type 1
CBG	Corticosterone-binding globulin
cDNA	Complimentary Deoxyribonucleic acid
CeA	Central amygdala
CEB	Cytoplasmic extraction buffer
ChIP	Chromatin Immunoprecipitation
CINC-1	Cytokine-induced neutrophil chemoattractant-1
CLOCK	Circadian Locomotor Output Cycles Kaput

CLPS	Colipase
CNS	Central nervous system
CORT	Corticosterone
CPM	Counts per minute
CRE	cAMP response element
CREB	cAMP response element binding protein
CRH	Corticotrophin releasing hormone
CRHR1	Corticotrophin releasing hormone receptor 1
CRTC2	CREB regulated transcription coactivator 2
CRY	Cryptochrome
DAX-1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DEX	Dexamethasone
DMH	Dorsomedial hypothalamus
DNA	Deoxyribonucleic acid
DSM-5	The Diagnostic and Statistical Manual of Mental Disorders, 5th edition
DST	Dexamethasone-suppression test
DTT	Dithiothreitol
DV	Dorsoventral
EDTA	Ethylenediaminetetraacetic acid
ER	Oestrogen receptor
ER-α	Oestrogen receptor alpha
FKBP4	FK506 Binding Protein 4
FKBP5	FK506 Binding Protein 5
FKBP51	FK506-binding protein 51
FKBP52	FK506-binding protein 52
FST	Forced swim test
GABA	Gamma-Aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoid
GH	Growth hormone

GILZ	Glucocorticoid-induced leucine zipper
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GSK3β	Glycogen synthase kinase 3 beta
HBC	2-hydroxypropyl-β-cyclodextrin
hnRNA	Hetero-nuclear ribonucleic acid
HPA	Hypothalamic-pituitary-adrenal
hrANXA1	Human recombinant ANXA1
Lat	Lateral
IL	Infralimbic
I.P.	Intraperitoneal
ISH	In situ hybridization
I.V.	Intravenous
LH	Luteinising hormone
LSD	Least significant difference
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MC2R	Melanocortin 2 receptor
MeA	Medial amygdala
MEB	Membrane extraction buffer
mEPSCs	Miniature excitatory postsynaptic currents
MNase	Micrococcal nuclease
MpFC	Medial prefrontal cortex
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NEB	Nuclear extraction buffer
Neuro D1	Neurogenic differentiation 1
nGRE	Negative Glucocorticoid response element
NMDA	N-Methyl-D-aspartic acid
NR3C1	Nuclear Receptor Subfamily 3 Group C Member 1

NTS	Nucleus tractus solitarii
Nurr1	Nuclear receptor related 1 protein
Nur 77	Nuclear receptor related 77 protein
NurRE	Nuclear receptor response element
OVX	Ovariectomised
PCR	Polymerase chain reaction
pCREB	Phosphorylated cAMP response element binding protein
PER1	Period circadian protein homolog 1
PFC	Prefrontal cortex
PKA	Protein kinase A
PL	Prelimbic
POMC	Pro-opiomelanocortin
PPIase	Peptidyl-prolyl isomerase
PR	Progesterone receptor
PVDF	Polyvinylidene fluoride
PVN	Paraventricular nucleus
PVT	Paraventricular thalamic nucleus
RCF	Relative centrifugal force
REM	Rapid eye movement
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RT-qPCR	Real-time quantitative polymerase chain reaction
SAFit	Selective antagonist of FKBP51 by induced fit
S.C.	Subcutaneous
SCN	Suprachiasmatic nucleus
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SF-1	Steroidogenic factor 1
SGK1	Serine/threonine-protein kinase
SHR	Steroid hormone receptor
SML	Squirrel monkey lymphocytes

SNP	Single nucleotide polymorphism
SSC	Standard saline citrate
StAR	Steroidogenic acute regulatory protein
STUB1	STIP1 homology and U-Box containing protein 1
TBST	Tris buffer saline with 0.05% Tween 20
TEBP	Telomere End Binding Protein
TPR	Tetratricopeptide repeat
TSST	Trier social stress test
TULP1	Tubby-related protein 1
VPG	Vesicular phospholipid gel
vSUB	Ventral subiculum
V1BR	Vasopressin receptor 1 B
WT	Wild type
qPCR	Quantitative polymerase chain reaction

Chapter 1 – Introduction

1.1. The hypothalamic-pituitary-adrenal axis and glucocorticoids

Glucocorticoids (GCs) are steroid hormones produced in vertebrate species in response to circadian cues and stress. When an organism (animal or human) is exposed to a physiological or psychological stressor, the subsequent increase in GCs enhances the chances of survival by preparing the body to cope with the stressor. The stress response is initiated by the activation of various afferent neuronal pathways, including those originating from the limbic structures of the brain (1). This response is very rapid, and GCs are synthesised and secreted from the adrenal gland within minutes upon exposure to a stressor. The activation of the hypothalamic-pituitary-adrenal (HPA) axis following stress results in increased attention, cognitive functions, and arousal, improved motor reflexes, a decrease in sexual arousal and appetite, and increased pain tolerance. The HPA axis activation also changes cardiovascular output, metabolism, and immune system regulation (2, 3). The main active GC in humans is cortisol, and in most rodents, including rats and mice, it is corticosterone (from here on referred to as CORT).

Glucocorticoids have a wide range of effects in the body and their signalling is essential for survival, with mutations in the glucocorticoid receptor (GR) in mice resulting in death within hours of birth (4). Glucocorticoids are perhaps most known for their anti-inflammatory properties and this has led to the development of several synthetic GCs currently used in the clinic. Further, GCs are involved in metabolic processes (5, 6) and cortisol is elevated in patients with insulin resistance, associated with hyperglycaemia and fatty liver (7, 8). Although some effects of synthetic GCs are widely characterised, the potential to provide therapeutics targeting a distinct mechanism of the HPA axis could minimise side effects, provide a disease-specific therapy, and more effective treatment.

1.1.1. HPA axis activity

Glucocorticoids are synthesised from cholesterol in the adrenal glands following the activation of the HPA axis. The HPA axis includes the paraventricular nucleus (PVN) of the

dorsal hypothalamus, the anterior pituitary gland, and the zona fasciculata of the adrenal cortex. The PVN is activated by other brain areas, both by circadian input from the retina via the suprachiasmatic nucleus (SCN) and by physiological and psychological stressors via limbic areas, the brain stem, and the frontal cortex. The PVN contains three major types of neurones, dorsomedial parvocellular neurones, dorsolateral magnocellular neurones, and autonomic parvocellular neurones. The dorsomedial parvocellular neurones comprise mainly corticotrophin-releasing hormone (CRH) expressing neurones, but also arginine vasopressin (AVP) expressing neurones and CRH and AVP are co-expressed in some. When the parvocellular neurons of the PVN are stimulated by neuronal input, they release CRH and AVP (9-11)(figure 1.1 A). Corticotrophin-releasing hormone is produced in these neurones and stored in vesicles in the axon terminals projecting into the median eminence. Afferent activation of CRH neurones causes depolarization and subsequent calcium influx which ultimately results in the fusion of CRH vesicles with the cell membrane and exocytotic release of CRH proximal to the pituitary gland portal vessels (12). Activation of CRH neurones also initiates the transcription of CRH, with increased messenger RNA (mRNA), followed by *de novo* synthesis of the CRH peptide. This process is initiated by an intracellular increase in cyclic adenosine monophosphate (cAMP), which is induced by PKA activation. Cyclic AMP enhances the binding of phosphorylated CREB (pCREB) to the cAMP response element (CRE) on the CRH promoter and ultimately results in enhanced transcription of the CRH gene (13).

The CRH and AVP travel via the portal vessels to the corticotropic cells in the anterior pituitary gland where they bind CRH receptor1 (CRHR1) and AVP receptor 1B (V1BR), respectively (14, 15). This induces enhanced transcription of pro-opiomelanocortin (POMC), which is further cleaved to produce to adrenocorticotropin-releasing hormone (ACTH) and ACTH is then stored in vesicles (12, 16). The binding of CRH to corticotropic cells also triggers the activation of PKA and cAMP which results in the phosphorylation of ion-channels and subsequent calcium influx and ACTH release by vesicular exocytosis (17). ACTH is secreted in the systemic circulation and reaches the adrenal glands where it binds its cognate receptor, the MC2R on zona fasciculata cells in the adrenal cortex (figure 1.1. A). The binding initiates a series of genomic and non-genomic events that ultimately results in the production of CORT. As CORT is lipophilic it cannot be stored in vesicles and has to be synthesised *de novo* and this process occurs within minutes of ACTH binding the MC2R (18, 19).

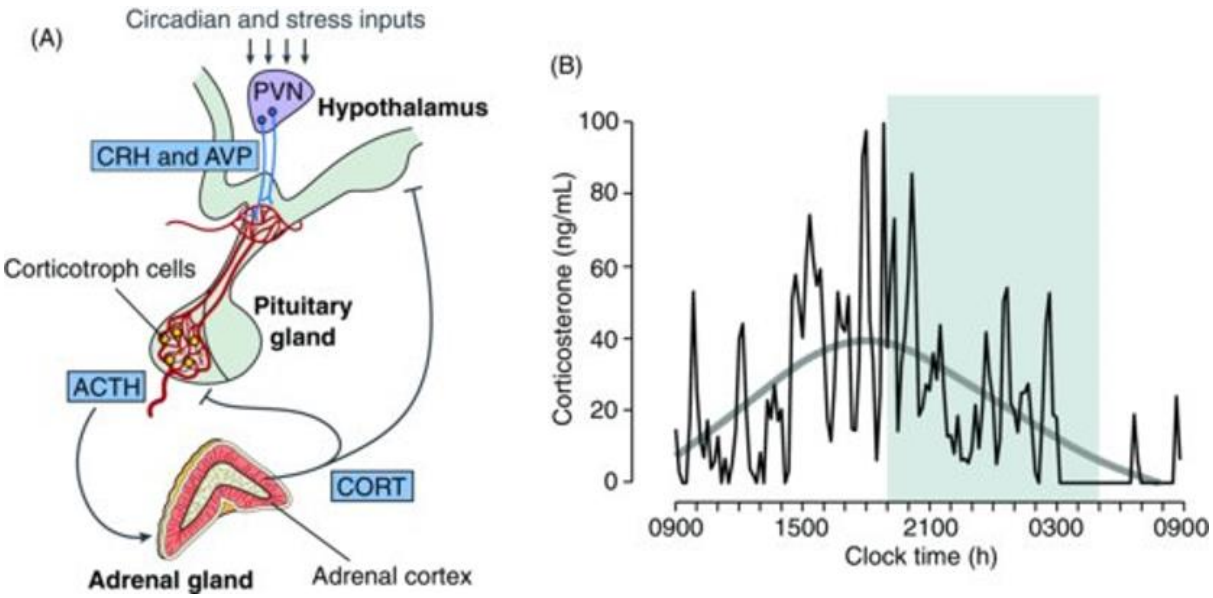

Figure 1.1. The HPA axis. *(A) The HPA axis is initiated by circadian cues and stress and activates CRH release from the PVN, which acts on the corticotrophs in the anterior pituitary gland to secrete ACTH. ACTH, in turn, activates GC synthesis and release from the adrenal glands. (B) A CORT profile from a male rat demonstrates the circadian pattern of CORT secretion, with increased levels before the onset of the dark (active) phase in the rat. From (20).*

1.2. Neuronal inputs to the HPA axis

The activity of the HPA axis is initiated and regulated by excitatory and inhibitory neuronal input to the PVN. This occurs via input from other brain areas following circadian stimulation or a stressor. These areas involve the SCN (discussed below), limbic areas such as the hippocampus and amygdala and other areas such as the prefrontal cortex (PFC) (21), and the thalamus (22-24). In this section, some of the neuronal inputs to the PVN will be described.

1.2.1. Hippocampus

The hippocampus projects to the PVN via the dorsal hippocampus, the lateral fornix, and the bed nucleus of stria terminalis (BNST), and exerts mainly inhibitory actions on the PVN (34). In rats, hippocampal ablation results in both elevated basal CORT concentrations and elevated CORT concentrations following some stressors, including restraint stress and the open field test (25-28). Further, lesion of the dorsal hippocampus or the lateral fornix results in dysregulation of the diurnal CORT rhythm and increases basal CORT concentrations (29, 30), whilst ablations of the whole hippocampus are required to observe an effect on stress-induced CORT secretion (31), suggesting a role for the hippocampus in regulating both basal GC secretion and following certain stressors.

In contrast to the dorsal hippocampus, the ventral hippocampus and the ventral subiculum (vSUB) are largely involved in the regulation of the CORT response following a stressor, and specific activation of the ventral hippocampus causes an increase in CORT concentrations (32). Lesions of the vSUB results in increased c-Fos and CRH peptide in the PVN in response to restraint stress, indicating activation of the HPA axis but does not affect basal CORT either in the morning or in the evening. Conclusively, the vSUB has an inhibitory effect on CORT secretions following physiological and cognitive stressors (28, 30, 33). The vSUB is an important region of the hippocampus as it is the point of output from the hippocampus to the PVN. Glutamatergic neurons in the vSUB project mainly to the BNST, medial preoptic area and dorsomedial hypothalamus, all areas that are rich in GABA-ergic neurons projecting directly to the PVN and exerting inhibitory actions (34).

1.2.2. Amygdala

The amygdala has an excitatory effect on the HPA axis activity and electrical stimulation of the amygdala results in elevated CORT secretion in rats (35), monkeys (36), and humans (37). Ablation of the amygdala causes reduced stress-induced CORT in response to odour stress (27) but not to ether (38). There are strong differences in the CORT response to certain stressors in the sub-regions of the amygdala and their functions. For example, the central amygdala (CeA) is sensitive to systemic stressors such as inflammatory stressors (39, 40), shown by a reduced stress response to these types of stressors, but not restraint stress

(41-43). In contrast, the medial amygdaloid nucleus (MeA) is sensitive to physical and psychological stressors such as noise, restraint and forced swim (39, 40, 44), and lesions of the MeA results in decreased CORT responses to restraint stress but not to an immune stressor (41). The CeA and the MeA project to the PVN indirectly via GABAergic input to the BNST (45), which in turn has GABAergic input to the PVN, suggesting that activation of the amygdala causes an activation of the PVN by a disinhibitory process. Moreover, the MeA projects to the preoptic area, which is highly expressing GABAergic neurons involved in inhibiting the HPA axis (46).

In addition, the basolateral amygdala (BLA) is involved in the response to psychogenic stressors, including forced swim and foot shock, but it is not activated following an immune stressor (39). Some studies have implicated the BLA in the role of chronic stress, and increased CRH mRNA in the CeA has been observed following synthetic GCs treatment and exposure to chronic stressors (47, 48). It has been suggested that the BLA is not involved in the regulation of acute stress, however, it is important in chronic stress. Indeed, chronically stressed animals with inactivated BLA showed increased HPA axis activity only in response to a novel stressor and not a familiar stressor (49).

1.2.3. Prefrontal cortex

Numerous studies have implicated the medial prefrontal cortex (MpFC) in the regulation of the stress response. Increased c-Fos and glucose utilisation in the MpFC is observed following numerous stressors (50-52). Indeed, lesion of the prelimbic (PL) area of the MpFC results in increased c-Fos expression in the PVN, as well as increased ACTH and CORT concentrations in response to restraint stress, but not a systemic stressor (53-55). The infralimbic (IL) region of the MpFC is involved in the regulation of immune stressors and other systemic stressors (56), and lesions of this region enhance the ACTH and CORT response to these stressors. Interestingly, ablation of the dorsal or ventral MpFC does not affect basal ACTH or CORT secretion (54, 55), suggesting that the MpFC is specifically involved in the stress-induced activation of the HPA axis (57). The PL and the IL cortex projects to several regions known to affect PVN activity, for example, the PL projects to the dorsal raphe involved in serotonergic-mediated activation of the HPA axis (58, 59). The PL also connects with the

BLA and the vSUB (58, 59). In addition, the IL projects to the BNST, CeA and the nucleus tractus solitarii (NTS), all of which in turn project to the PVN to regulate HPA axis activity. The IL also projects to the dorsomedial and lateral hypothalamus, inhibiting HPA axis activity (59-61).

In addition to areas described in detail above several other areas are also involved in the direct or indirect activation of HPA axis activity, including BNST, other nuclei within the hypothalamus itself, intra-PVN neurons, brain stem nuclei, and more (21). Thus, a wide range of brain areas are involved in regulating the PVN output and hence HPA axis activity. Interventions to try and alter GC secretion can potentially, therefore, target various brain areas.

1.3. Glucocorticoid and Mineralocorticoid Receptors

The various effects elicited by GCs occur by binding their cognate receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). These receptors belong to the nuclear receptor superfamily (62, 63). When inactive, both the GR and MR reside in the cytoplasm where they are associated in a complex with various chaperone proteins including the heat shock proteins hsp90, hsp70, and hsp23, and the two closely related immunophilins from the FK506-binding protein family, FKBP51 and FKBP52 (64, 65).

Studies in the rat in the 1980s revealed a difference in distribution between the GR and the MR (66, 67). The GR is widely expressed throughout the body and is involved in inducing CORT-mediated regulation of metabolic events and immune system functions, whilst peripherally the MR is expressed mainly in the kidney (68) and the heart (69). In the rat brain, MR is mainly expressed in the limbic system with high expression in the hippocampus and low expression in the amygdala. The GR is expressed in most brain areas, highly expressed and co-localized with MR in the hippocampus, and amygdala, and highly expressed in frontal brain regions and the pituitary gland (62, 66, 67).

Further, the MR has an approximately 10 fold higher affinity for CORT than do GR (66) and is therefore occupied when CORT concentrations are low; in the morning in rats and the trough of the ultradian pulses. On the other hand, GR has a lower affinity for CORT and is

therefore only occupied when CORT levels are high such as during the circadian peak, and after a stressor, when the CORT concentrations increase rapidly (66).

Due to their difference in distribution and occupancy, the MR and GR exert different effects on the body. In the periphery, MR in the kidney regulates water and salt balance. In the brain, tonic activation of MR, mainly in the hippocampus, is involved in the regulation of cognitive functions, mood and affect (62); it also relays sensory information to ensure an appropriate neuroendocrine and behavioural response. Concerning the role of MR in regulating HPA axis activity, intra-hippocampal injection of the MR antagonist RU28318 increases basal HPA axis activity both in the morning and evening and results in higher CORT concentrations compared to the vehicle-treated animals, following exposure to a novel environment. The increase in basal CORT following an MR antagonist demonstrates the effect of MR on tonic inhibition of the HPA axis, whereas the increase in CORT after a stressor is believed to be due to inhibited MRs in the limbic areas and hence leads to increased stress response (70). In addition to its peripheral effects on metabolism and immune system function, centrally active GR initiates the stress-mediated process and, in the PVN and the pituitary gland, the GR mediates the negative feedback mechanism of GCs. This negative feedback response is important in regulating CORT concentrations, and altered GR activity and subsequent changes in the GC negative feedback is implicated in psychiatric disorders (71).

Glucocorticoids are lipophilic and hence readily cross the plasma membrane. In the cytoplasm, free CORT can bind the GR and MR and subsequently causes the receptors to translocate to the nucleus where they regulate gene transcription of various target genes. Nuclear translocation of GR and MR is regulated by the binding of the receptor to the FK506-binding protein 51 and 52 (FKPB51 and FKBP52) (65, 72). The role of FKBP51 and 52 in regulating GR activity will be discussed further below.

1.4. HPA axis rhythms

1.4.1. HPA axis Circadian rhythm

The circadian rhythm in an organism refers to the daily biological variations over 24 hours. Circadian rhythms are conserved in organisms ranging from bacteria to mammals and are characterised by several processes including biochemical, physiological, endocrine, and behavioural, functioning to prepare the organism for changes in the environment in the light/dark cycle. In mammals, GCs synthesis and release are synchronised in a circadian pattern with the highest levels upon awakening, to prepare the organism for energy expenditure and to synchronise physiological processes in a daily rhythmical manner. In diurnal mammals such as humans, the highest levels of GCs occur in the morning, and in nocturnal mammals, including the rat and the mouse, this occurs in the evening. These circadian rhythms are synchronised by a master clock in the ventral hypothalamic SCN (73-75). The central regulation by the SCN is entrained by light exposure, which is perceived by the retina and relayed to the SCN via the retino-hypothalamic tract (76, 77).

Several 'clock genes' in the SCN form an oscillating system, including the circadian locomotor output cycles kaput (CLOCK), brain and muscle aryl hydrocarbon receptor nuclear translocator-like (BMAL1), period 1-3 (PER1-3), and cryptochrome 1-2 (CRY1-2) (78-80). These genes are transcribed in a rhythmic fashion resulting in a rhythmic oscillation of the proteins, controlled by negative and positive transcriptional and translational feedback mechanisms (81). In rodents, lesion of the SCN produces a disruption in circadian rhythms in body temperature, locomotor activity, and endocrine activity (82-85). Peripheral tissues, including the liver, kidney, lung, skeletal muscle, and the adrenal, also express clock genes with circadian variation in their activation, (75, 86-88). The SCN sustains its rhythm but the peripheral clocks are dependent on the circadian drive from the SCN to maintain their light/dark synchronisation (89, 90).

1.4.2. Regulation of HPA axis circadian rhythm

The PVN receives direct input from the SCN via the dorsomedial hypothalamus (DMH) (91, 92) and results in an overall circadian rhythm of CRH synthesis and secretion. This is turn ensures a circadian release of ACTH from the anterior pituitary gland (92, 93). In the adrenal

cortex, most of the steroidogenic proteins, for example, steroidogenic acute regulatory protein (StAR), are also expressed in a circadian manner, shown by variations in both mRNA and protein levels during the 24 hours (94-96). Circadian variations in both ACTH and adrenal steroidogenic proteins work synergistically to produce circadian rhythms of CORT secretion from the adrenal cortex.

The SCN contains AVP-expressing neurons that project to the PVN and are involved in regulating SCN output and hence important in regulating HPA axis circadian rhythms (97). Studies using micro-infusions of AVP or an AVP antagonist, in the DMH or PVN directly have shown an inhibitory effect of AVP on CORT secretion in the rat (98). This is further demonstrated by a decrease in AVP in the SCN neuron terminals in the DMH before the dark phase in the rat, and this is necessary for the circadian peak of CORT (99). Hence in nocturnal mammals such as the rat, the light entrains AVP release from the SCN to ensure low CORT levels during the circadian nadir (day time) (99). By contrast, in diurnal species such as humans, the light also induces AVP release from the SCN, however, in diurnal species, this AVP release has a stimulatory effect on the HPA axis. Hence, AVP can both stimulate or inhibit CRH release from the PVN, and this depends on activation of excitatory or inhibitory neuronal projections to the PVN which in turn regulate the CRH release (91, 92). However, there is as yet no evidence for direct connections between AVP-containing neurones in the SCN and the CRH-expressing neurons in the PVN (100, 101).

1.4.3. HPA axis Ultradian Rhythms

In addition to the rapid increase in CORT following an acute stressor, basal HPA axis activity exhibits high-frequency pulsatile dynamics, which make up the circadian rhythm of GC secretion. This circadian rhythm is characterised by a peak of GC secretion before the active phase, comprising the night in nocturnal animals such as rats and mice, and the morning in diurnal mammals such as humans (102, 103). The high-frequency pulsatile dynamics, so-called ultradian rhythms, have a variable amplitude of the GC pulses throughout the 24 hours and result in the overall circadian variations in GC concentrations (104, 105). The discovery of this ultradian rhythm in the rat was achieved with the help of an intra-adrenal gland microdialysis technique (104) and since then variations of an automated blood sampling

system have been used allowing plasma CORT to be measured in frequent intervals during physiological and pathological changes (105, 106).

In addition to the rat, ultradian rhythms of GCs have also been observed in sheep (107), monkeys (108), and humans (109, 110). Importantly, pulsatile patterns of ACTH secretion have also been demonstrated in rats (111, 112), sheep (113), and humans (109), and these ACTH pulses precede the pulses of CORT (104, 109). Thus a pulse in ACTH causes a subsequent pulse in CORT, which is slightly delayed due to the synthesis of CORT which cannot be stored in vesicles. The concentrations of plasma and ACTH and CORT can be measured via blood sampling from, for example, a jugular vein cannula, however, measurement of CRH concentrations must be done in the hypophyseal portal system. Such surgery is very stressful for rats, and this together with low sensitivity of CRH assays, has resulted in a limited amount of studies investigating CRH release pattern *in vivo*. Nevertheless, it has been shown that CRH is secreted in a pulsatile manner in free-moving rats (114, 115) and sheep (116).

The pulsatile pattern of CORT secretion is crucial for the responsiveness in the target tissue (117). Gene expression analysis in various GC target tissues shows that when the same dose of GCs is delivered either through a constant infusion or in a pulsatile manner, this results in a different pattern of gene regulation (118). Further, it has been shown that the pulsatile secretion of CORT from the adrenal gland is reflected in pulses of CORT in the hippocampus (119). Moreover, pulsatile CORT results in pulsatile PER1 transcription in cell lines and rat liver (118) and it has been shown that each pulse of CORT causes a rapid, but transient, increase in GR translocation and subsequent PER1 transcription in the hippocampus (120). Although further research is needed to elucidate the role of pulsatile CORT for gene regulation in target tissues current evidence indeed suggests a crucial role for pulsatile CORT secretion.

1.4.4. Regulation of ultradian rhythms

The regulation of circadian rhythmicity by the SCN is well characterised (121), and lesions of the SCN in rodents results in loss of circadian variations in CORT secretion as demonstrated by increased levels in the morning and hence a flattened curve of CORT concentration (122-124). Interestingly, Waite and colleagues showed that in rats with SCN

lesion, or rats kept in constant light, the ultradian pulsatility is maintained even though the circadian rhythm was disrupted (85). This intriguing study suggested that the regulation of ultradian pulsatility might be separate from the central control of circadian rhythmicity of CORT concentrations. This is in contrast to the long-standing belief that the ultradian rhythms are under hypothalamic control. This is the case for other hormones such as luteinising hormone (LH) and growth hormone (GH) that are secreted in a pulsatile pattern from the pituitary gland as a consequence of a pulsatile hypothalamic releasing factor (125-127).

Corticotrophin-releasing hormone is the hypothalamic releasing factor for ACTH and it has been demonstrated that CRH secretion follows a pulsatile pattern (114, 115, 128). It was therefore assumed that the hypothalamic pulse generator of pulsatile CRH generated the pulsatile ACTH release from the anterior pituitary gland and subsequent pulsatile CORT secretion. However, in the rat, CRH has a pulse frequency of approximately 3 pulses per hour, compared to the hourly pulses of ACTH and CORT (fig 1.2.). Moreover, the hypothalamic pulse generator was further questioned when it was demonstrated that blocking CRH by using a CRH antagonist, had no significant effect on ACTH pulsatile pattern in regards to pulse frequency, length, or amplitude (129). Similarly, in sheep, the ultradian pulses of CORT are maintained without PVN-mediated input to the pituitary gland (116).

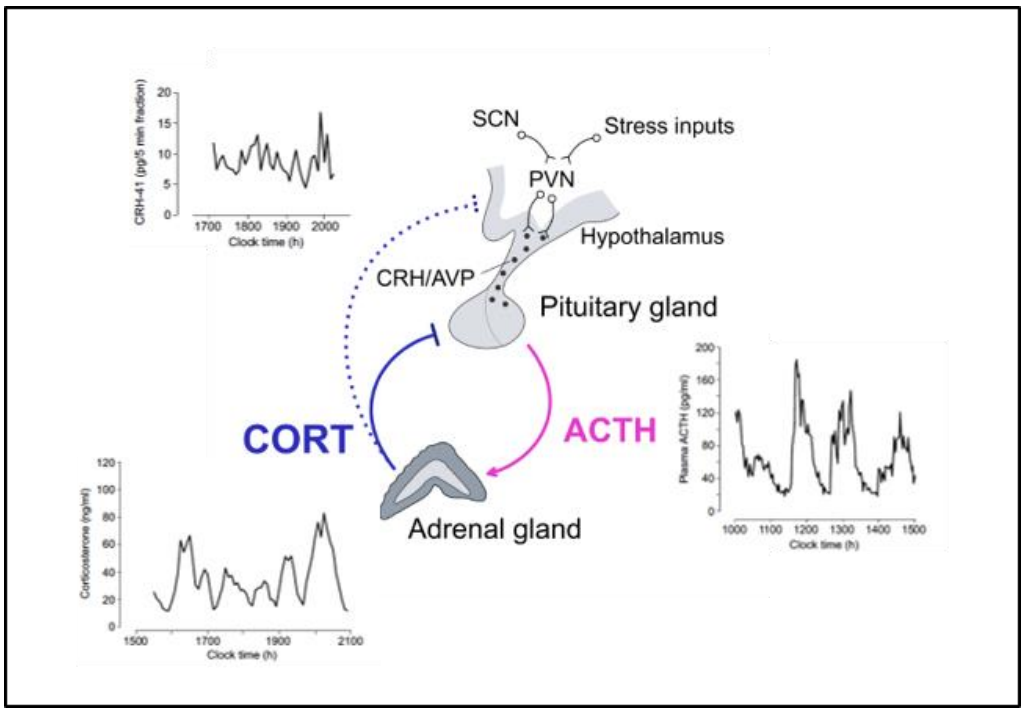

Figure 1.2. Ultradian rhythms of the HPA axis activity and glucocorticoid secretion. *The PVN is activated by SCN input and from limbic regions such as the hippocampus and amygdala, and rapidly releases CRH stored in vesicles. This, in turn, causes the release of ACTH from the anterior pituitary gland, which activates CORT synthesis and secretion from the adrenal glands. Note that the ultradian pulse frequency is approximately 3 pulses per hour for CRH, while for ACTH and CORT the pulse frequency is approximately 1 pulse per hour. From (130).*

Mathematical modelling combined with *in vivo* studies in the rat has provided further evidence for a sub-hypothalamic control of CORT ultradian pulsatility which is dependent on dynamic interactions of the pituitary gland and the adrenal gland (131, 132). The hypothesis of this pituitary-adrenal gland pulse generator comprises a positive feedforward by pituitary gland ACTH which drives adrenal gland CORT synthesis and secretion, and the CORT-mediated negative feedback turns off pituitary gland ACTH release. The secretory time delay in the adrenal gland following stimulation by ACTH, and the delay in the negative feedback mechanism at the pituitary gland, resulting in ultradian pulses of CORT secretion. The CORT and ACTH pulses have been well characterised both in the rat (111, 131, 133, 134) and in humans (109). As CORT is lipophilic and cannot be stored in vesicles, the CORT synthesis in the adrenal cortex occurs de novo following each pulse of ACTH stimulation. Aiming further

to elucidate the generation of ultradian pulsatility, mathematical modelling of that HPA axis system has shown the CRH concentration is more important than the pulsatile pattern of CRH for subsequent pulses of ACTH and CORT (132).

Furthermore, this mathematical model supports a sub-hypothalamic pulse generator indicated by predictions of a self-sustaining pituitary-adrenal gland loop of ACTH and CORT pulses (132). The model predicts a self-sustaining ACTH/CORT loop only when levels of CRH are 'physiological' i.e. equivalent to the circadian peak of HPA axis activity. The self-sustaining loop is lost when CRH levels exceed this, for example at constantly high CRH as observed *in vivo* during a stressor (105, 135)(figure 1.3). The validity of the model predictions was tested *in vivo* by administering rats a constant dose of CRH at a low (0.5 mg/h) or higher concentrations (1.0 mg and 2.5 mg/h) and measuring CORT concentrations at high-frequency intervals by automated blood sampling (figure 1.3.). The results showed that indeed a constant CRH infusion at a low concentration results in pulsatile CORT secretion, while a higher dose of CRH does not (85). Importantly, the resulting pulsatile pulses of CORT following the low CRH dose resemble those occurring during normal physiological conditions in the active phase in untreated rats (figure 1.3.). Furthermore, the study also showed that in the rats receiving the low CRH dose, a pulse of ACTH was followed by a pulse of CORT (131). Mathematical models can, therefore, be useful tools for examining pulsatile systems and when combined with *in vivo* data, support hypotheses.

The hypothesis of a sub-hypothalamic pulse generator was further supported by *in vitro* evidence in cultured rat pituitary gland cells. When the cells were treated with constant CRH this resulted in ACTH secretion, which was then subject to inhibition by the resulting CORT. Further, it was shown that this inhibitory effect of CORT on the pituitary gland ACTH secretion was independent of gene transcription, instead suggesting a non-genomic membrane-associated GR mechanism (136).

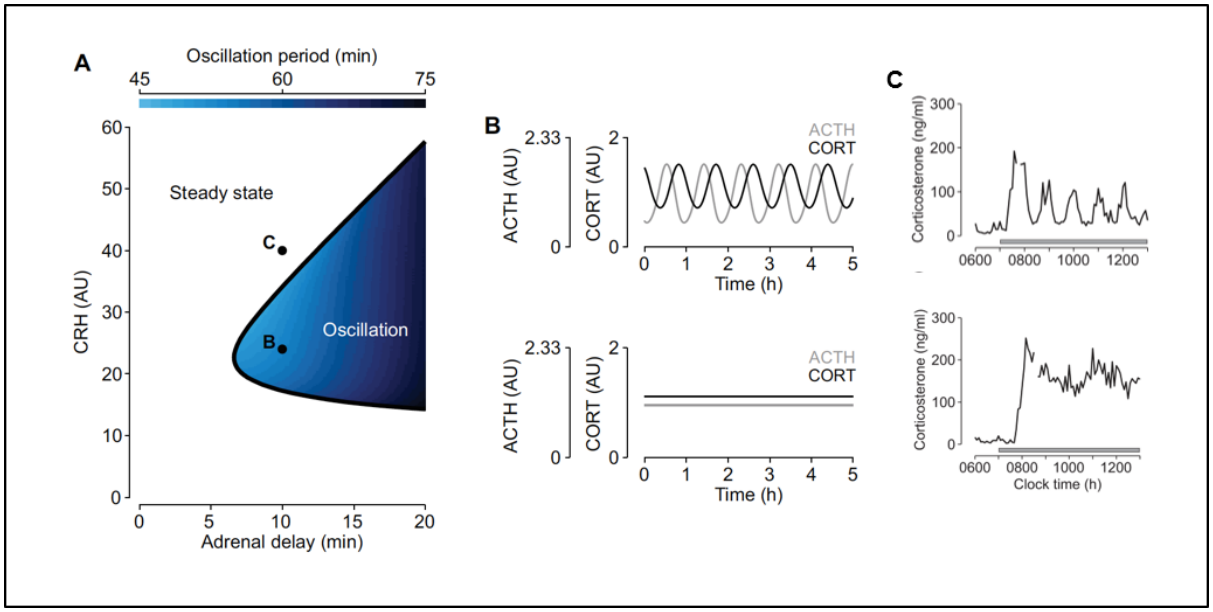

***Figure 1.3**. **The pituitary-adrenal model of ultradian rhythms.** Mathematical modelling and experimental data show HPA axis dynamics to variations in CRH drive. (A) A computed two-parameter bifurcation diagram predicts the difference in CORT oscillations in response to variable CRH drive. Point B illustrates CRH drive within the pulsatile region, which results in subsequent pulsatile CORT. Point C illustrates a point where the CRH drive is outside the pulsatile region and results in constant CORT response. (B) The top diagram shows a pulsatile CORT response to a CRH drive that is within the pulsatile region (point B in (A)). The bottom diagram shows a constant CORT response to a CRH drive, which is outside the pulsatile region (point C in (A)). (C) The top graph shows experimental data from rats that were injected with a constant dose of CRH at a concentration corresponding to a CRH drive within the pulsatile region which caused pulsatile CORT response. The bottom graph shows experimental data from rats injected with a higher dose of CRH, which is corresponding to a CRH drive outside the pulsatile region and resulted in constant CORT response. Reproduced with permission from Spiga et al. 2014 (20).*

1.5. Rapid non-genomic effects of glucocorticoids in the brain and pituitary gland

There has been a long-standing notion that GCs are only responsible for the long term, delayed responses to stress, and that neurotransmitters and peptides, such as noradrenaline, cause the acute effects of stress (137). Although this is partly correct, GCs have a wide range of effects, some that occur within minutes and are hence too rapid to be genomic (138).

The PVN of the dorsal hypothalamus is a crucial component of the HPA axis. The PVN neurons have a high expression of GR (66). Activation of GR in the PVN by CORT induce the negative feedback mechanism to inhibit HPA axis activity. The negative feedback occurs both in a delayed, genomic fashion (139) and in a rapid, non-genomic way (140, 141). Interestingly, one study showed that this rapid negative feedback occurred following the intra-PVN injection of dexamethasone conjugated to the membrane-impermeable BSA (DEX-BSA) (142). The same group showed that this was dependent on the cannabinoid receptor type 1 (CB1) (143). This indicates that in the PVN, GCs can rapidly reduce HPA axis activity by a membrane-associated mechanism involving endocannabinoid signalling. Further progress in this area was made by Tasker et al. with their investigation on the miniature excitatory postsynaptic currents (mEPSCs) in PVN neurons (144). An mEPSC can be used to measure neuronal activity and is a measure of postsynaptic activity, caused by the release of a presynaptic glutamate vesicle (145). Importantly, the presynaptic activity determines the frequency of these mEPSCs. Tasker et al. showed that high doses of CORT (100nM to 1uM) or dexamethasone result in a decrease in the frequency of miniature excitatory postsynaptic currents (mEPSCs) in PVN neurons (144, 146). This effect occurred rapidly, within 5 minutes, and did not subside when the CORT was removed, suggesting that the effect is prolonged. Furthermore, the GC-meditated effect on mEPSCs was not affected by GR or MR antagonists (144, 147). Current evidence suggests that this rapid negative feedback mechanism in the hypothalamus is initiated by GC binding to a G-protein coupled receptor (GPCR) at the postsynaptic terminal. This causes the production of the cAMP –PKA pathway which ultimately results in the production of anandamide (AEA) and 2-arachidonoylglycerol (2-AG). The AEA and 2-AG activate presynaptic CB1 receptors which inhibit the release of glutamate vesicles from the presynaptic terminal (148).

As described in section 1.2. the hippocampus, amygdala, and prefrontal cortex provide limbic input to the PVN, hence control HPA axis activity, and are important for the adaptation to stressful stimuli (149). The limbic areas are involved in processing information to create a memory of the stressful event. In particular, the hippocampus is involved in contextual memory formation of the stressful event. Both GR and MR are highly expressed in the hippocampal subfields, although predominantly MR is expressed in the CA3 (66).

Similar to the hypothalamus, the neurons in the hippocampus exhibit spontaneous activity of mEPSCs. Studies using hippocampal slices from mice have shown that the administration of CORT increases mEPSCs frequency in the CA1 region within 5 minutes of CORT administration (150, 151), opposite to the effects in the hypothalamus. Similar effects are seen in the dentate gyrus (152). It has been shown that these rapid effects are independent of gene transcription events and that they involve a receptor in the plasma membrane (150). Furthermore, these rapid effects in the hippocampus occur at a 10-fold lower dose of CORT compared to the hypothalamus. The effect is present in GR-knockout mice, but not MR-knockout mice suggesting that these rapid effects are MR-mediated (150). Furthermore, it was suggested that the MR is located at the plasma membrane and has a lower affinity to CORT than the cytoplasmic receptor (150), and will be activated when CORT concentrations rise quickly such as after stress (153). Further studies showed that this membrane-bound MR is present at the presynaptic membrane (151).

In addition, a rapid increase in spine density in the hippocampus has been observed following exposure to GCs, and this effect is GR-dependent (154). Other, GC-mediated rapid effects in the hippocampus have been observed and are both MR and GR-independent, suggesting a role for a novel membrane receptor. These GC effects on the hippocampus include stimulation of the inhibitory projections from the hippocampus, a rapid increase in extracellular excitatory amino acids (155), LTP (156), NMDA-mediated neurotoxicity (157), and inhibition of NMDA signalling (158, 159).

Emotional experiences usually create strong memories and this is, to a large part, dependent on the amygdala. The amygdala is implicated in emotional processing, typically associated with fear and anxiety, cued learning, and memory processing and is activated following a stressful event (160, 161). Both MR and GR are widely expressed throughout the different amygdala subnuclei (66) and GCs are implicated in the amygdala-dependent

memory processing (160, 161), thus making it sensitive to stressful events. Further, the amygdala receives input from the adrenergic system and both noradrenaline and adrenaline are secreted in the amygdala following stress. In mice and rats, intra-amygdala injections of noradrenaline in the BLA, but not the CeA, enhances stress-induced memory consolidation. Conversely, blocking the β-adrenoreceptor impairs memory consolidation (160, 161). Similarly, the administration of a GR agonist following an inhibitory avoidance paradigm enhances memory consolidation (162), while a GR antagonist impairs memory consolidation (163). This suggests that both the adrenergic system and the HPA axis are involved in amygdala-dependent memory formation following emotional arousing events. It is suggested that the effects of GC in enhancing memory formation is due to the adrenergic signalling. One study showed a rapid increase in noradrenaline in the amygdala following CORT administration, followed by enhanced memory formation (164). Further, membrane-associated MR and GR have been found in amygdala neurons (165, 166). One study showed that GRs are located at the membrane of dendritic spines and post-synaptically (165), suggesting a role in the synaptic plasticity of emotional events.

The PFC is an important area in decision-making and behaviour and has projections from the hippocampus and the amygdala (167). Few studies have investigated the rapid effects of GCs in the PFC. Studies using synaptosomes showed an enhanced uptake of glutamate following CORT administration (168). *In vivo* corticosterone or CORT-BSA injection, in the insular cortex of the PFC result in enhanced recognition in an object recognition test (169). This effect was not seen when a GR antagonist was co-administrated, indicating a membrane-mediated effect of the GCs involving GR. The same study also showed that PKA and CREB were involved in the memory facilitation.

The important role of rapid GC-mediated negative feedback in the anterior pituitary gland was first reported in the 1970s and 1980s (170-172). The GR is highly expressed in the pituitary gland, while MR levels are low (173). These rapid negative feedback mechanisms are non-genomic and result in the inhibition of ACTH release being observed within 1 min of subsequent CORT administration (172). The rapid effects on inhibition of ACTH release were only seen when the CORT concentrations were increasing but not when the CORT concentrations were high already (170, 174, 175). The mechanism of ACTH inhibition will be discussed in more detail in section 1.7.

1.6. Inhibition of CRH release

CRH-producing neurons are located in the PVN of the dorsal hypothalamus. Corticotrophin-releasing hormone is stored in the axon terminals in the external zone of the median eminence. When the CRH neurons are stimulated by a calcium influx the CRH vesicles fuse with the membrane and are secreted, via exocytosis, close to the pituitary gland vasculature (12). Simultaneously, stimulation of the CRH neurons results in increased CRH transcription and subsequent increase in de novo synthesis of CRH peptide. Transcription of CRH is stimulated following activation of the cAMP/PKA pathway and involves the activation of pCREB which binds the CRE in the CRH promoter (176, 177). CRH transcription is also regulated by CREB binding to CREB regulated transcription coactivator 2 (CRTC2), a co-regulator of transcription (178, 179). This de novo synthesis is vital to replenish the intravesicular storage of CRH and for the system to remain responsive (180).

There is strong evidence that CORT may inhibit CRH synthesis and secretion (181-183). The CRH expression in the PVN of adrenalectomised rats is significantly higher than in intact animals (184). In addition, in ADX animals, even a mild stressor was significant to increase the CRH mRNA whilst this was not seen in the intact animals (185-187). Furthermore, mice with dysfunctional GRs in the PVN have increased CRH mRNA accompanied by hypersecretion of ACTH and CORT (188). The GC-mediated effects on CRH-expressing PVN neurons are partly mediated by the effects of GCs on brain regions with afferent projections to the PVN, those include the hippocampus, the prefrontal cortex, and the amygdala. However, CORT also has a direct negative feedback on the CRH-neurons in the PVN mediated by the GR (189, 190).

1.6.1. Non-genomic and genomic mechanisms regulating CRH synthesis and secretion

Non-genomic. The rapid, non-genomic effects of CORT in the PVN involve a putative membrane GR and involves endocannabinoid signalling (146). Whole-cell patch-clamp recordings in the hypothalamus have demonstrated that CORT decreases glutamatergic transmission post-synaptically in PVN neurons. This is, as previously mentioned, dependent on a post-synaptic G-protein coupled receptor and a pre-synaptic CB1 (144). Administration of dexamethasone conjugated to bovine albumin serum (DEX-BSA) inhibits HPA axis activity, an effect that is blocked by a CB1 receptor antagonist (142, 143). However, it has been shown

that GR KO mice lack rapid GR-mediated negative feedback, suggesting that the classical membrane GR may be involved in addition to a membrane GR. Indeed, it has been proposed that classical GR may regulate the expression of the putative membrane GR (191).

Genomic. The CORT-mediated negative feedback of CRH transcription is complex and not fully elucidated. No classical GRE was found in the CRH gene promoter in an *in vitro* study using AtT-20 cells with transfected human CRH. However, the same study demonstrated that GR binding to an nGRE in the CRH promoter results in repression of transcription (192, 193). Furthermore, other studies show that CORT mediates its inhibitory effects on CRH transcription by preventing CREB from binding to the CRE in the CRH promoter, and this is mediated via GR-CREB protein-protein interactions (194, 195). Amongst other factors, activation of CREB (pCREB) is regulated by CREB Regulated Transcription Coactivator 2 (CRTC2). Unstimulated CRTC2 is found phosphorylated in the cytoplasmic compartment and, upon stimulation, CRTC2 is de-phosphorylated by various kinases, including salt-inducible kinase (SIK), and translocates to the nucleus. Once in the nucleus CRTC2 can enhance pCREB transcriptional activity (196, 197). Recent evidence suggests that the GR may be involved in regulating the phosphorylation and translocation of the CRTC2 in the hypothalamus. Dexamethasone administrations, and exposure to stress both cause increased phosphorylation of CRTC2 in mice (198). Finally, there is evidence that in addition to the effects on transcriptional regulation of CRH, CORT may affect the stability of the CRH mRNA (199).

1.7. Inhibition of ACTH release

Corticotrophs in the anterior pituitary gland are stimulated by CRH binding the CRHR1 resulting in activation of ACTH synthesis and release via both genomic and non-genomic events (17).

1.7.1. Non-genomic and genomic mechanisms regulating ACTH synthesis and secretion

Non-genomic. In the corticotrophs, ACTH is stored in vesicles and when stimulated by CRH is secreted rapidly. The CRH-mediated activation of the CRHR1 results in increased cAMP

levels and PKA activation which subsequently cause the phosphorylation of ion-channels and enhanced calcium influx which in turn causes ACTH vesicles to fuse to the membrane and are secreted via exocytosis (17). Glucocorticoids may inhibit this release by interfering with the electropotential properties of the corticotrophs. Corticotrophs have spontaneous firing and when stimulated with CRH this causes increased bursts and frequency of action potentials (200). Bursting enhanced calcium influx, therefore, may induce the release of the ACTH vesicles (201-203). Large-conductance calcium- and voltage-activated potassium (BK) channels are required for bursting firing in corticotrophs (200, 204, 205), and it is thought that the BK channels are therefore important for ACTH secretion. In murine corticotrophs, it has been shown that CORT administration leads to both reduced spontaneous firing and reduced bursts firing induced by CRH. In addition, the same study showed that this CORT-mediated decrease in burst firing caused reduced ACTH release and that this is BK channel-dependent (200). The mechanism of how CORT is mediating these effects is still unclear however it is suggested to be via PKA-dependent phosphorylation of the BK channels (206, 207).

In addition to electropotential properties of the corticotrophs, Annexin1 (ANXA1) may also regulate ACTH vesicular release. Annexin1 is produced in folliculostellate cells in the pituitary gland and bind its putative receptor, the formyl peptide receptor. It is thought that ANXA1 causes the inhibition of CRH-mediated ACTH release by rearranging the cytoskeleton hence affecting vesicular transport, via a signalling pathway activated by cAMP and calcium influx (208). Glucocorticoids, in turn, can rapidly regulate ANXA1 activity by inducing translocation to the membrane from the cytoplasm by post-translational modifications (208). In addition, GCs enhance ANXA1 transcription which is a slower process and involves genomic events, however, it is important to replenish the cytoplasmic levels of the ANXA1 following CORT-induced translocation (209).

Genomic. The genomic actions of CRH on ACTH production involve transcription of POMC, the precursor of ACTH. Corticotrophin-releasing hormone binding to the CRHR1 causes activation of the cAMP and subsequent PKA-mediated phosphorylation of CREB which binds the POMC promoter and stimulates transcription (210, 211). Furthermore, CRH –induced cAMP and PKA signalling activate the calcium/calmodulin kinase II and the MAPK pathway to increase expression of Nur77/Nurr1 (212, 213). Nur77 binds the NurRE in the POMC promoter and enhances transcription of the POMC gene (213) hence increases ACTH production.

Glucocorticoid-meditated ACTH inhibition at a genomic level involves GR binding to the nGRE at the POMC promoter, which inhibits transcription (214, 215). Further, it has been demonstrated that the GR may prevent Nur77-mediated POMC transcription (213, 216). Recently, it has been suggested that this is mediated via NeuroD1. NeuroD1 is known to play a role in the regulation of POMC transcription (217) and when CORT is absent, the NeuroD1 binds the E-box on the POMC promoter to cause transcription (218). When CORT concentrations rise, NeuroD1 expression is decreased (217) and therefore there will be less POMC expression and a subsequent decrease in ACTH levels.

In summary, there are various ways that GCs and GR activity can affect both CRH and ACTH secretion. Although the exact mechanisms through which GR-mediated negative feedback regulates CRH and ACTH expression and secretion, and how GR activity is regulated, are not fully understood. Further insight into these mechanisms will be useful to further unravel the regulation of HPA axis activity.

1.8. Adrenal Gland Negative Feedback

Several studies support the idea of an intra-adrenal gland negative feedback system where CORT inhibits its own synthesis by directly affecting the steroidogenic pathway in the adrenal gland. The CORT-mediated effect on steroidogenesis has been shown both *in vitro* (219, 220) and *in vivo* (221, 222), even though the mechanism is not fully elucidated. Studies using dexamethasone indicate that GCs can inhibit steroidogenesis by enhancing the expression of Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (DAX-1), a negative regulator of the rate-limiting protein for steroidogenesis – StAR (223). Furthermore, similar to POMC regulation, CORT may inhibit StAR transcription by GR-mediated interaction with Nur77 (224, 225). Moreover, CRTC2 is not only involved in CRH regulation but is also involved in the transcriptional activity of steroidogenic genes in the adrenal gland, mediated via CREB activated transcription (226, 227). One study showed that CORT inhibits the nuclear translocation of CRTC2 (198), a crucial part of its transcriptional function. Whether this occurs in the adrenal gland and is part of an intra-adrenal gland feedback system is yet to be discovered, however, it is an interesting speculation.

Both mathematical modelling and *in vivo* studies have provided some evidence for non-genomic and rapid effects of CORT on its synthesis and/or secretion (133, 134). It is suggested that CORT can regulate the activity of some of the steroidogenic proteins, however, the exact mechanism is yet to be elucidated. However, experimental work in the rat shows that a pulsatile pattern of ACTH administration causes a rapid increase in intra-adrenal gland CORT and subsequent activation of the GR as measured by phosphorylation at Serine 211 (133). There is no evidence for CORT-mediated regulation of the steroidogenic protein activity yet, however, CORT has been shown to regulate the PKA by regulation of the catalytic subunit of the protein (228). As previously discussed, some of the non-genomic effects of CORT in the anterior pituitary gland are mediated by inhibition of ANXA1 (229), and ANXA1 is also present in the adrenal gland where it is also regulated by CORT (230). Isolated adrenal glands from ANXA1-null mice have a larger CORT response to ACTH compared to the adrenal glands from wild-type mice (230). This suggests that in addition to the regulation of ACTH secretion, ANXA1 may be involved in an intra-adrenal gland negative feedback loop by regulating the synthesis or secretion of CORT.

A deeper understanding of the role the adrenal glands play in the regulation of CORT secretion is crucial for further development of synthetic GCs used in the clinic. It is not currently known to what extent intra-adrenal gland GR-mediated negative feedback is involved in regulating CORT secretion.

1.9. Sexual dimorphism in the HPA axis

1.9.1. Rodent studies

Numerous studies have demonstrated sexual dimorphism in HPA axis activity in both rodents and humans (231), characterized by hyperactivity of the HPA axis in females resulting in overall higher CORT levels. Several rodent studies have compared the circadian rhythm of ACTH concentrations in the basal state in males and females and the rhythms seem to be similar in both sexes (232-234). Although one study revealed a flatter ACTH pattern throughout the day in male rats while the females showed a circadian rhythm with higher levels before and during the dark phase. Following gonadectomy, this pattern was somewhat reversed with a slight delay in the increase in ACTH in the males (235). Further, numerous

studies indicate that there is no difference in the basal concentrations of ACTH between males and females (236-238).

Further studies involving gonadectomised female and male rats show a difference in basal CORT concentrations measured over a 24-h period (239, 240)(fig 1.4). Sham female and sham male rats were compared to castrated female and castrated male rats. As expected, sham females had higher amplitude of CORT pulses compared to male rats (60.1 and 23.9 ng/ml CORT, respectively), as well as higher frequency of the pulses (1.03 and 0.49 pulses per hour, respectively), with a higher number of total pulses in the females compared to males (24.3 and 11.5, respectively). Interestingly, the opposite was seen in the castrated animals; the castrated males displayed significantly higher pulse amplitude than the castrated females (63.1 and 29.1 ng/ml CORT, respectively), significantly higher frequency of pulses (0.95 and 0.56 pulses per hour, respectively), and significantly higher number of total pulses (22.6 and 13.1, respectively) (239). The same study investigated the mRNA expression of GR in the PVN and showed that castrated males had significantly lower GR mRNA levels than those observed in sham males, sham females had significantly lower levels than sham males and ovariectomized females. This suggests that GR is involved, plausibly lower levels of GR result in reduced negative feedback and higher CORT concentrations. Additionally, CRH mRNA levels in the PVN were significantly higher in sham females compared to sham males, and ovariectomized females and castrated males had higher CRH mRNA levels than sham males, sham females, and ovariectomized females. The same pattern was seen for the POMC mRNA levels in the anterior pituitary gland (239). In another study, the gonadectomised rats were given hormone replacement with either testosterone or dihydrotestosterone in the males or 17b-oestradiol in the females. These hormone replacements reversed the effects seen by the gonadectomy in both sexes, in both CORT concentrations and the pulsatile pattern (240).

In contrast to basal sex differences in the HPA axis activity, sex differences in stress-induced ACTH and CORT suggest both a central and a peripheral involvement. Rodent studies show higher ACTH concentrations in females following various psychological and physiological stressors (236, 238, 239, 241-243). One study found that female rats had a significantly higher ACTH response to alcohol administration compared to males, irrespective of the oestrous cycle stage, although the ACTH response was higher in females in the proestrus and oestrus stage compared to females in the diestrus stage (236). Seale and

colleagues showed that three hours post LPS injection, sham males and ovariectomized females had similar levels of ACTH whereas castrated males and sham females had similar, and significantly higher concentrations of ACTH. These effects of gonadectomy were reversed when the animals were given hormone replacement, dihydrotestosterone or testosterone, and 17b-oestradial, for males and females, respectively (239, 240).

Numerous studies have reported higher absolute CORT concentrations in females compared to males following various stressors. For example, following a psychological stressor such as restraint stress, the CORT concentrations are higher in the females at the end of the stressor (238, 244-248). These differences are seen in various stressors such as the forced swim test, foot shock, alcohol administration, immobilisation, novel environment, ether and noise stress to name a few (236, 237, 239, 242, 249-253), and females show higher CORT responses to both predictable and unpredictable stressors (254). In addition, frequent sampling studies have shown that the CORT concentration in females, following a stressor, rises more quickly and stays elevated for longer, compared to males (241, 250, 251, 254, 255). Moreover, Seale and colleagues further investigated the effects of gonadectomy in rats. The sham and castrated females and males were subjected to noise stress and an immune stressor, on two different occasions. Post-stress CORT concentrations following the noise stress were significantly higher in the castrated males, compared to sham males as well as sham females and castrated females. Further, the LPS-mediated increase in CORT resulted in increased CORT concentrations in castrated males 90 min post LPS injection compared to sham males, castrated females, and sham females (239). In the same study, three hours post-LPS administration, the levels of PVN AVP and CRH, and anterior pituitary gland POMC mRNA were measured in all four groups. Castrated males had significantly higher levels of all three genes compared to intact males, whilst lower levels were observed in the ovariectomized females compared to intact females. Moreover, GR mRNA expression in the PVN was significantly lower in castrated males compared to intact males and increased in ovariectomized females compared to intact female rats (239). These changes were reversed by the administration of respective sex hormones (240). These observations indicate a pivotal role of sex steroids in regulating HPA axis activity and suggest that androgens such as testosterone, activate GR transcription whilst oestrogens inhibit GR transcription.

To date, there are currently no reports of any ER response elements (EREs) or AR response elements (AREs) on the GR promoter, although it is a plausible mechanism for how sex hormones may affect GR expression and activity.

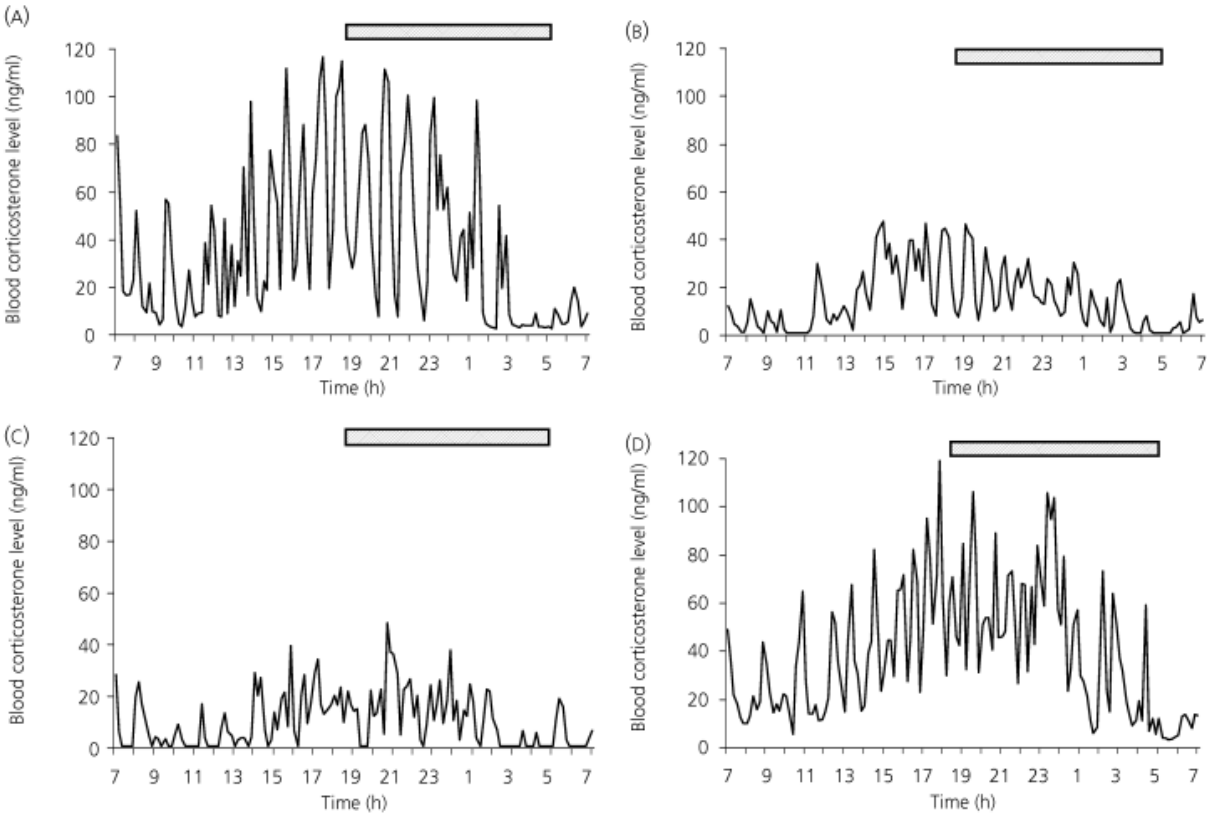

***Figure 1.4*. Male and female CORT profiles.** *Blood corticosterone concentrations (ng/ml) measured every 10 min over 24 hours in basal conditions (from 07.00h to 07.00h) in a sham female rat (A), a sham male rat (B), an ovariectomized female rat (C), and a castrated male rat (D). The grey bar indicates lights off (19.00h to 05.00h). From* (239).

1.9.2. Human studies

Compared to the rodent studies on the sexual dimorphism in the HPA axis, the human studies show more variable results, presumably due to the variation in age and demographics of the subjects, and the methodologies used. In human studies, both plasma and saliva hormone samples are used and only free CORT is measured in the saliva samples, while both free CORT and CBG-bound CORT are detected from the plasma samples. A large number of

stressors can be applied however the most standardised is the Trier Social Stress Test (TSST) which involves the most reliable tests in activation of the HPA axis: motivated performance, uncontrollability, and a social threat (256). The TSST involves a preparation period followed by a speech and a mental arithmetic task. Studies using the TSST or other similar tests have found higher ACTH response in males and similar CORT concentrations in both sexes (257-260). One study also investigated the effect of oral contraceptives and found that there was no difference in total plasma CORT between males and females following the TSST, irrespective of the menstrual cycle stage, however, women using oral contraceptives had higher CORT concentrations than the males and other females (261). Furthermore, the saliva CORT concentrations were higher after the TSST in males and females in the luteal phase compared to the females in the follicular phase and on oral contraceptives (261). Tests with social interactions and rejection elicit higher free CORT concentrations in women, while men showed a greater increase in CORT following verbal and mathematical tests (262). These studies suggest that in humans, females show higher HPA axis activity following social rejection specifically, while men are more sensitive to most other psychological stressors.

Moreover, for physical stressors, most studies show no sex difference or higher HPA axis activity in women. No sex difference in CORT was observed following intensive endurance exercise (263, 264) however when high-intensity exercise was preceded by dexamethasone administration, to activate GC negative feedback, the women had higher CORT concentrations (265), suggesting that there is less GC-mediated negative feedback in women following a physical stressor. Other forms of physical stress such as heat were found to result in increased ACTH and heart rate in women, while the CORT concentrations were similar in both women and men (266). Similarly, subsequent to one hour of exposure to cold, ACTH and CORT concentrations were significantly higher only in females (267).

Similar to physiological stressors, pharmacological activation of the HPA axis results in higher hormone concentrations in women. Activation of the HPA axis by the administration of exogenous ovine CRH results in a larger increase in ACTH concentrations, and a longer CORT response in women compared to men (268). Another study with human CRH administration found no difference in the ACTH response, although a greater response in women was seen when CRH was co-administered with AVP (269). During the dexamethasone-CRH test (DEX-CRH test) the patients are given a dose of dexamethasone the night before the investigation,

followed by the administration of CRH the following day. The DEX-CRH test, therefore, assesses the GC negative feedback regulation. Interestingly, women show higher CORT concentrations following the Dex-CRH test compared to men, suggesting a lower GC negative feedback activity in women. (270).

Conclusively, sex differences in HPA axis activity in humans are dependent on the type of stressor the subject is exposed to. Certain psychological stressors, such as social rejection (262), and pharmacological stressors (260) activate the HPA axis more in women, while most other psychological stressors affect men more (260). This is perhaps due to a difference in the brainstem or limbic structures in men and women.

1.9.3. Central and peripheral molecular mechanisms of sexual dimorphism of the HPA axis

With regards to molecular differences in the HPA axis in males and females, CRH and AVP expression in the PVN have been studied to elucidate any potential differences in central activation of the HPA axis. In rats, basal expression of PVN CRH is higher in females compared to males (238, 241, 271). Similarly, some studies show that following a stressor such as a footshock or an immune stressor, females show a greater increase, and a longer duration of PVN CRH expression (239, 241). However, these results are inconsistent with other studies indicating higher post-stress CRH expression in the PVN of males following restraint stress (238, 248). Thus, the differences in post-stress CRH expression increase appear to be largely dependent on the type of stressor. In humans, men have been shown to have more CRH-expressing neurons in the PVN (272) which could account for the increased HPA axis response following certain stressors in men. Studies in rats have shown no clear difference in basal AVP mRNA expression in the PVN (238, 273-275) but a greater increase of AVP following stress in females (238, 239). Finally, no apparent difference in PVN neuronal activation, measured by c-Fos induction, has been established following stress in rats (238, 248, 250, 274).

Glucocorticoid-mediated negative feedback is a pivotal mechanism for terminating the stress response and this is induced by circulating CORT binding to GR both centrally and peripherally. Moreover, CORT binding to the MR, mainly in the hippocampus, executes a tonic inhibition of the HPA axis (276). Although not many studies have investigated the sexual

differences in GR and MR expression and binding in males and females, a few important observations have been made. The affinity of CORT for MR is markedly higher in male rats than in female rats (REF). The implication of this is that in male rats the MRs are predominantly occupied in the basal state, when CORT levels are low, whilst in females both MRs and GRs will be occupied. Thus, in female rats the MRs are partially unoccupied even at basal levels, and might not be filled until relatively high levels of CORT are reached (277). Studies show that progesterone may bind the GR, which causes a reduced binding of CORT to the GR (278) which might contribute to reduced GR-mediated GC negative feedback. Furthermore, oestrogen administration in OVX females for four days reduced the MR binding in the pituitary gland by 50%, and this effect was not present in males (279). It has also been shown that oestrogen treatment reduces GR binding in the hippocampus specifically (280). This inhibitory effect of oestrogen on GR and MR binding is predominantly seen at pro-oestrus, when oestrogen levels are high and result in reduced negative feedback and higher basal levels of CORT. In contrast, testosterone has stimulatory effects on GR binding, anatomically distinct to AVP containing neurons, resulting in inhibition of AVP-mediated ACTH release from the anterior pituitary gland (reviewed in (277). Studies investigating GR and MR expression have mainly focused on the hippocampus and hypothalamus, and most report no major differences in expression (277).

Seale and colleagues investigated the expression of GR mRNA in the sub-regions of the hippocampus, and the anterior pituitary gland, in sham-operated males and females, and gonadectomised males and females. In the anterior pituitary gland, the expression of GR mRNA was lower in sham females compared to sham males and castrated males. In the hippocampus, there was no difference in GR mRNA across the groups (239). These results perhaps suggest that lower CORT binding to MR and GR in females, and lower GR expression in the anterior pituitary gland in females, could result in decreased GC-mediated negative feedback in females and subsequently higher CORT concentrations.

There is a strong line of evidence that indicate sex differences in the adrenal gland's capacity for CORT production. In rodents, females have heavier adrenal glands if expressed as a ratio of total body weight (281). More specifically this difference in size is due to a larger volume of the zona fasciculata of the adrenal cortex, the part of the adrenal gland where CORT synthesis occurs, hence females have a higher capacity for CORT synthesis than do

males (281, 282). The secretion capacity of CORT from the adrenal glands is higher in female rodents (242, 283), thus the same concentration of ACTH results in higher CORT secretion in females compared to males.

In addition to sex differences in central and peripheral activation of the HPA axis, there are differences in the clearance and free CORT in males and females. Female rats have (282) shorter biological half-life of CORT (242, 284), due to a higher metabolism of CORT in the liver (285, 286). Corticosterone and cortisol are only biologically active in the target tissues when not bound to CBG (110). With approximately two-fold higher levels of CBG in adult female rats compared to male rats (287-291), this could contribute to reduced GC-mediated negative feedback in females.

1.10. HPA axis, glucocorticoids, and depression

The Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5) criteria for major depression states that the patient must have experienced the symptoms as a change from their previous state and that the symptoms are present over 2 weeks. At least one of the patient's symptoms must be either depressed mood experienced almost every day, or a loss of interest in all activities. The patient must also exhibit four of the following symptoms: significant alterations in appetite and/or weight (not due to dieting), insomnia or hypersomnia, psychomotor alterations; agitation or retardation, loss of energy or fatigue, feelings of guilt and worthlessness, indecisiveness or impaired ability to think, and frequent thoughts about death or suicide (292).

Once the criteria for major depression are met, the DSM-5 divides depression into four subtypes or subcategories: postpartum depression, melancholic depression, atypical depression and catatonic depression (292). Interestingly, patients with different subtypes of depression show differences in HPA axis activity and their circadian profiles of the symptoms (293). In the majority of severely depressed patients, a hypercortisolism with intact circadian rhythmicity is often present, partly due to increased hypothalamic CRH secretion (294). This is particularly true for those patients with melancholic depression exhibiting most of their severe symptoms in the morning (292, 295, 296). Melancholic depression is characterised by anhedonia, low mood and worse mood in the morning, early awakening, weight loss,

disturbed psychomotor abilities, and guilt. These symptoms have been observed more in elderly patients than in younger patients (297-299). In addition, men are more likely to present with melancholic depression than women (300). Further epidemiological data regarding gender differences in depression have shown that the higher prevalence of depression described in women is only present for the atypical subtype of depression (301).

Compared to patients with Cushing's syndrome, characterised by elevated cortisol and a loss of circadian rhythmicity, melancholic depressed patients do not develop the clinical symptoms of Cushing's despite similarly high circulating levels of GCs (292). This is likely to be due to a GC resistance in depressed patients. Even though depressed patients exhibit circadian rhythmicity of GC secretion, the circadian nadir is shorter and more pulsatile compared to healthy controls (302). Further, these patients have sleep disturbances; less deep sleep (slow-wave) and reduced latency to REM sleep (302). When the depressed patient is remitted, both the hypercortisolism and the alterations in circadian rhythms disappear, suggesting these effects are state-dependent rather than trait-dependent (295).

On the other hand, atypical depression is characterised by a blunted circadian rhythm of cortisol secretion (292). Patients with atypical depression present with disturbances in the evening, such as fatigue, lack of energy, hypersomnia, and significant weight gain as a consequence of significantly increased appetite (292). In addition, patients suffering from atypical depression often exhibit variations in mood, leaden paralysis (feeling of limbs being weighed down), and sensitivity to rejection (292). The reduced circadian variation in cortisol concentrations is caused by a reduced HPA axis activity, the opposite of melancholic (typical) depression. This reduced HPA axis activity in atypical depression is caused at least partly by reduced activity of the CRH secreting neurons in the PVN, resulting in a subsequent lower circulating cortisol concentration (293). In addition to the gender differences in the prevalence in atypical depression, the atypical depression subtype has been associated with earlier onset in the sufferers, a chronic disease burden, and poor outcome to tricyclic antidepressants, suggesting specific biological features (303, 304).

The initial evidence for a dysregulated HPA axis in some depressed patients emerged in the 1980s, where it was shown that depressed patients had elevated plasma CORT and reduced GC negative feedback (305). Further studies evaluating HPA axis activity in depression have used various ways of measuring circulating basal cortisol or used studies

involving challenging the HPA axis or the negative feedback mechanism. Studies using the former approach have used techniques including measuring cortisol in urine, blood, or saliva samples. The more sophisticated of those include frequent sampling over 24 hours for cortisol and ACTH. The challenging tests mostly involve stimulating the negative feedback mechanism with the GR agonist, dexamethasone (306), the so-called dexamethasone-suppression test (DST), or a combination of DEX and CRH (DEX-CRH) to investigate pituitary gland function (307). The DST results in suppressors, with a normal response to the DEX, and non-suppressors, with impaired negative feedback mechanisms. In the melancholic subtype of depression, around 50 % are non-suppressors in the DST (308). Further, increased ACTH and cortisol responses have been reported following the DEX-CRH test in patients with major depression, however differences in subtypes were not investigated (307). This increased DEX-CRH response suggests decreased sensitivity in the pituitary gland negative feedback. Further, is has been shown that in elderly patients with depression the relationship between cortisol and depression was U-shaped and the elderly with lowest CORT concentrations scored higher on the depression scale than patients with higher CORT (309).

These studies highlight the differences in HPA axis activity, and symptoms, amongst patients diagnosed with depression. To be able to understand and treat the altered HPA axis activity in these patients, a deeper understanding of the HPA axis, and especially how GC ultradian rhythms are regulated, is needed.

1.11. The FK506-binding protein 51 and 52

The FK506 binding proteins 51 and 52 (FKBP51 and FKBP52) were discovered over 20 years ago and first identified as part of the steroid receptor complex (310, 311). Since then, both proteins have gained interest in their ability to regulate these steroid receptor activities and the effects on hormone signalling. Due to their effect on endocrine regulation, both FKBP51 and FKBP52 have been investigated as potential therapeutics for endocrine-related pathologies including prostate cancer, breast cancer, metabolic disorders, and stress-related diseases (312).

Both FKBP51 and FKBP52 bind the hsp-90 of various steroid hormone receptor (SHR) complexes via the C-terminal tetratricopeptide repeat (TPR) domain (313). FKBP51 and

FKBP52 share 70% similarity in structure and both contain a peptidyl-prolyl isomerase (PPIase) domain, and two N-terminal domains FK1 and FK2, in addition to the TPR domain. FKBP51 regulates SHR activity by regulating the translocation of the receptors and hence their genomic activity. FKBP52 has been shown to promote receptor translocation and hence the transcriptional activity of the GR (65), progesterone receptor (PR) (314), and the androgen receptor (AR) (315), but not the oestrogen receptor (ER) or the MR (65). In contrast, FKBP51 is a negative regulator of most SHRs (72) including the GR and MR. These two FKBPs compete for the binding of the SHR and hence if FKBP51 is overexpressed, the SHRs will be less regulated by the FKBP52 (65). Some studies also indicate that hormone-binding affinity is significantly decreased when the SHR is bound to FKBP51 (316).

Further, it is suggested that FKBP51 attaches to the receptor complex at the later stages of the chaperone cycle, and shows preferential binding to hsp-90 complexes that are bound to a ligand-free SHR, for example, the GR (317-320). The mechanism of the preferential binding to ligand-free GRs and other SHRs is not yet clear. In addition to the inhibitory properties on nuclear translocation, FKBP51 had been shown to decrease the hormone-binding affinity to its ligand (321, 322). It is not clear whether this is due to direct inhibitory effects of the FKBP51, or indirect effects by preventing the binding of FKBP52. Indeed, *in vitro* studies have shown FKBP51 reduces the rate of nuclear translocation of GR by preventing FKBP52-mediated translocation of the receptor (323).

The FKBP51 gene FKBP5 has a functional GRE, and thus, an intracellular short feedback loop exists whereby less FKBP51-induced GR translocation and binding to the GRE on the FKBP5 gene induces more FKBP51 expression and thus reduced GR translocation (324-326). In addition to its role in GR and MR translocation FKBP51 can also regulate the activity of certain kinases, including AKT (327) and GSK3β (328), which in turn can regulate GR activity (329, 330).

Although it is well-known that FKBP51 inhibits GR translocation hence GR genomic activity (65), it is not established to what extent this affects the role of GR in regulating GC-mediated negative feedback. It is currently unknown how central and peripheral FKBP51 may differ in function and actions.

1.12. FKBP51 and glucocorticoid resistance

The FKBP51 gained a lot of interest when it was found that there was a link between FKBP51, increased cortisol concentrations and GC resistance in squirrel monkeys (322). It was shown that squirrel monkey lymphocytes (SML) had increased levels of FKBP51 and that this was implicated in the GC resistance and high circulating levels of cortisol seen in those monkeys (322). The increase in circulating cortisol compensates for the decreased affinity of the GRs as a result of the increased expression of the FKPB51 (331). The transcriptional activity of GR in the squirrel monkeys is similar to those of human GR as demonstrated by *in vitro* studies with the two different GRs transfected into cells (332). The effect of the FKBP51 on GR binding was assessed and it was found that in the squirrel monkeys the increased expression of FKBP51 did indeed cause a substantial decrease in GR binding (332). Hence it is the increase in FKBP51 and the resultant decrease in GR binding which results in GC resistance and increased cortisol concentrations in squirrel monkeys. This led to the intriguing hypothesis that the FKBP51 could be implicated in mental disorders caused by an increased HPA axis activity in humans.

1.13. FKBP51 and polymorphism – link to mental disorders

In humans, a breakthrough in the research of mental illnesses came when Binder and colleagues were the first to demonstrate a relationship between functional polymorphisms in the FKBP5 gene and the response to antidepressants in patients with major depressive disorders. The study investigated the correlation between 57 single nucleotide polymorphisms (SNPs) in the genes encoding for known co-chaperones to the GR: BAG1, STUB1, TEBP, FKBP4 and FKBP5 (333) and the GR itself (NR3C1), and the outcome of antidepressant treatment following 2 and 5 weeks of hospitalization and at remission in patients with major depressive disorders (n=294) and matched controls (n=339). The results showed a correlation between three SNPs (rs1360780, rs1334894, and rs755658) in the FKBP5 gene with the treatment outcome of at least two out of three time points of recovery or remission in the depressed patients (334). To investigate whether this correlation was solely due to the FKBP5 or if it involved adjacent genes, further analysis investigated 27 SNPs in the 288kb surrounding the FKBP5 gene—this comprised FKBP5 and the three surrounding

genes (TULP1, FLJ25390, and CLPS). An analysis of linkage equilibrium was performed and further analysis for the correlations of antidepressant treatment and SNPs in these regions was conducted.

With regard to the SNP rs1360780 (with two possible alleles; C/T), a study showed a significant interaction between allele and outcome of antidepressant treatment after two weeks of treatment (334). The patients were genotyped into CC, CT or TT and the outcome after two weeks were non-responders: CC:25; CT:16, TT:1, and responders: CC:14; CT:24; TT:5. Notably, the rs3800373 (G/A) showed a trend of correlation (p=0.053), with AA allele patients displaying a higher rate of response to the treatment. Nevertheless, the SNP rs1360780 was selected for further analysis, including physiological and molecular studies. Firstly, the analysis showed that TT patients had a significantly higher number of previously recorded depressive episodes, more than twice as many as the CC and CT genotypes. Thus, although the patients with the TT genotype are more responsive to treatment, they are also more prone to develop depressive episodes, suggesting a strong involvement of a dysregulated HPA axis. Further molecular analysis of the FKPB51 level in lymphocytes revealed higher protein levels of FKBP51 in the TT patients compared to CC and CT genotypes. However, when the peripheral blood monocytes were analysed for FKBP5 mRNA levels the results showed no difference between the three genotypes. This suggests that increased FKBP51 protein levels are due to increased translation or increased protein stability.

Furthermore, the ACTH and cortisol levels in the patients were examined; the TT genotypes had lower ACTH concentrations compared to the CR and CC genotypes following a DEX-CRH test, however, there was no difference in the cortisol concentrations between the three genotypes, although all three genotypes displayed significantly higher CORT concentrations compared to healthy controls. The morning after the DEX-CRH test the cortisol levels were significantly lower in all three genotypes compared to their respective basal cortisol however there was no difference between the TT and the CC or CT genotypes. Interestingly, although no difference in FKBP5 mRNA levels or plasma cortisol levels were present in the three genotypes in healthy controls, the TT genotypes showed a stronger positive correlation between FKBP5 mRNA levels and plasma cortisol, which was significantly different from the CC and CT genotypes (334).

Another group performed correlation studies of the SNP rs1360780 and found an association between the less common TT genotype and depression in men but not women when investigating the prevalence of major depression, mixed anxiety depression and dysthymia (335) suggesting a sexual dimorphism in FKBP51-mediated regulation of HPA axis activity. Lekman et al., performed a large comparison study of three FKBP5 SNPs; rs1360780 (C/T), rs4713916 (A/G), and rs3800373 (A/C) across three different populations: all, white non-Hispanic, and black, investigating the prevalence of depression, remission, and response to treatment. Both genotype- and allele-wise comparison tests were performed on cases and controls, remitters and non-remitters, and responders and non-responders. The genotype TC in the rs1360780 was overrepresented in cases (46% vs 38%) in the white non-Hispanic group. Further, in the genotype-based association test, there was a significant association between the rs4713916 (A/G) and remission in the white non-Hispanic group. In the allele-wise comparison, the A allele was significantly over-represented in the remitters when all groups were combined (336).

In addition to the effects of certain SNP genotypes of the FKPB5 and association with the various effects on mood disorders, some studies have investigated the effect on the TSST. Ising and colleagues investigated the effect of genotype in common FKBP5 SNPs, the ACTH and cortisol concentrations, and self-reported anxiety during the TSST. Patients with the TT genotype of the rs1360780 SNP, the GG genotype of the rs3800373 SNP, and the AA genotype of the rs4713916SNP all had higher plasma cortisol concentrations during the recovery period in their first and second TSST. Additionally, the GG genotype of the rs3800373 SNP and the AA genotype of the rs4713916SNP also had higher self-reported anxiety during the recovery of the second TSST (337).

The association between the genotypes of the rs1360780 SNP and cortisol response to the TSST was tested in one study with combined men and women in healthy controls and patients with remitted major depression. Surprisingly, there was no effect of the genotypes in the remitted group. There was, however, a strong significant effect of genotype in the healthy controls. Subjects with the TT genotype had higher cortisol concentrations 15 minutes following the TSST. During the baseline and the test, there was no difference in cortisol concentrations. Furthermore, the same study investigated the levels of FKBP5 mRNA during the test in both groups and all three genotypes. In the healthy controls, the CC genotype only

showed induction of FKBP5 mRNA following the TSST and had significantly higher FKBP5 mRNA at the 70 minute post-stress time point, whilst there was a blunted response of FKBP5 mRNA induction in the TT and CT healthy controls (338). The above studies have shown a strong link between FKBP5 genotypes and psychiatric disorders, however, they did not show a clear association between FKBP5 expression and depression, and did not investigate the expression of FKBP5 in the brain. However, a few studies have indeed shown an increased FKBP5 mRNA expression in patients with MDD. For example, Mamdhani and colleagues showed a significant 2.36 fold increase of FKBP5 mRNA in the hippocampus in patients with MDD compared to controls in a post-mortem study (339).

The studies above suggest that FKBP51 has a strong role in psychiatric disorders, plausibly by affecting the GR activity and hence the GC-mediated negative feedback. Although this is yet to be fully elucidated, it provides a strong hypothesis for further research to unravel the complexity of the molecular mechanism underlying psychiatric disorders with altered HPA axis activity.

1.14. FKBP51 and rodent studies

1.14.1. Overexpression of FKBP51 in the mouse

Studies in mice overexpressing FKBP51 in the basolateral amygdala (BLA) induced anxiety-like behaviour in the mice (340). An adenovirus vector overexpressing the FKBP5 gene was injected bilaterally in the BLA and post-mortem gene expression analysis showed a threefold increase in FKBP5 mRNA expression compared to vehicle-treated mice (339). The FKBP51 overexpressing animals showed significantly reduced entries and time spent in the open arms of the elevated plus maze test, indicating an increased anxiety phenotype in these animals (340). Similar results were seen when the FKBP51 was overexpressed in the central amygdala, suggesting that both areas of the amygdala are involved in anxiogenic behaviour. Further, viral-mediated overexpression of FKBP51 in the dorsal hippocampus, dentate gyrus and CA1, did not affect anxiety-related behaviour, indicating that this is not mediated by the hippocampus (340). The amygdala projects to the PVN and causes an increase in ACTH release and hence subsequent CORT release, believed to be involved in some of the anxiety-related behaviours (21).

Glucocorticoid actions in the amygdala have been demonstrated to be involved in regulating a positive feedback loop that ultimately potentiates the activity of the HPA axis. Additionally, studies in mice have shown decreased GR expression and increased anxiety-like behaviour which is reversed by viral-mediated restoration in GR levels (341). This suggests that increased amygdala FKBP51 expression, or decreased GR expression, induces anxiety in mice. This suggests that GCs and the GR in the amygdala are involved in regulating HPA axis activity and that an increase in FKBP51, or decrease in GR, and subsequent decrease in nuclear GR induced increased HPA axis activity and anxiety.

It is not currently known whether overexpression of FKBP51 in other brain areas influences HPA axis activity and further research in this area will be useful to establish the role of central FKBP51 activity.

1.14.2. FKBP51 Knock-out studies in the mouse

In contrast to the FKBP51 overexpression studies, a knockout mouse model was developed to study the effects of decreased FKBP51 levels, and whether this altered the HPA axis activity. In male FKBP51 knock-out (51KO) mice, there was no difference in basal morning CORT levels, or 15 min after an FST. However, 90 min after the FST, CORT levels were significantly lower in the 51KO mice. The other group of 51KO mice was subjected to 21 days of social defeat stress and morning CORT levels were significantly lower in the stressed 51KO group compared to the stressed wild type (WT) group. Similarly, CORT concentrations 15 min post-FST and 90 min post-FST were significantly lower in the stressed 51KO mice. The mice were tested for behavioural effects using the FST, the elevated plus maze, and the open field test. There was no significant effect of genotype except for the time spent swimming in the FST where stressed 51KO mice showed increased time spent swimming (342). The same tests were performed in female 51KO mice, with similar results, although there was a significant decrease in morning CORT levels in non-stressed animals as well as stressed animals. This might be because female mice have higher basal CORT levels, whereas in male mice, especially in the morning, CORT levels are low and therefore the effect of genotype is not seen (343).

These data suggest that significantly reducing the levels of FKBP51 enhanced the ability to terminate the stress response in both female and male mice. However, in the above studies the CORT concentrations were only measured at two time points in the basal conditions, and at one time point following the stressor. A complete 24-hour profile of basal CORT following altered FKBP51 expression or activity has not yet been established.

1.15. FKBP51 antagonists: SAFit1 and SAFit2

The two natural ligands for FKBP51, FK506 and Rapamycin, both have inhibitory effects on FKBP51 activity and bind the FK1 domain of the FKBP51 (344). However, the main effects of FK506 and Rapamycin are, respectively, calcineurin inhibition and mTOR inhibition hence are immunosuppressive and limit their use in living organisms. Therefore, a search for non-immunosuppressant FKBP51 inhibitors began, however, this was for a long time hindered by the structural similarity to FKBP52, as the FK506-binding sites are conserved (345). Selective inhibition is crucial due to the opposite effects of FKBP51 and FKBP52 on glucocorticoid signalling (346).

A breakthrough came when a research team at Max Planck Institute of Psychiatry lead by Dr Felix Hausch developed two potent ligands for selective inhibition of FKBP51. The two ligands, SAFit1 and SAFit2 (short for selective antagonist of FKBP51 by induced fit) showed over 10,000-fold selectivity for the FKBP51 over FKBP52. Moreover, both SAFit1 and SAFit2 have a 10-fold selectivity for the related FKBP51 protein FKBP12. Importantly the SAFit1 and SAFit2 have a 100-fold higher affinity for the FKBP51 (347). The two SAFit compounds bind the 506-binding pocket but lack the effector domain that mediates the binding to calcineurin or mTOR and are therefore not immunosuppressive (347). Although the SAFit1 and SAFit2 are the best FKBP51 inhibitors published to date, the physicochemical properties are not optimum for CNS-directed drugs (348) and further optimisation is required. Nevertheless, after an injection of 10 mg/kg (i.p.) in male mice, it was shown that SAFit2 has a half-life of 9.7 hours and SAFit1 2.5 hours. The C_{max} (maximum concentration) for SAFit2 was 2094.8 and was reached after 0.3 hours (T_{max}). In contrast, the C_{max} for SAFit1 was 1383.0 ng/ml and the T_{max} 1.0 hours (347). This suggests that SAFit2 reached a higher concentration compared to SAFit1, and reached the maximum concentration faster than SAFit1. In addition, the brain:

plasma ratio for SAFit2 is 16.7%, while for SAFit1 it is <0.5%, demonstrating that SAFit2 penetrates the brain and SAFit1 does not (347).

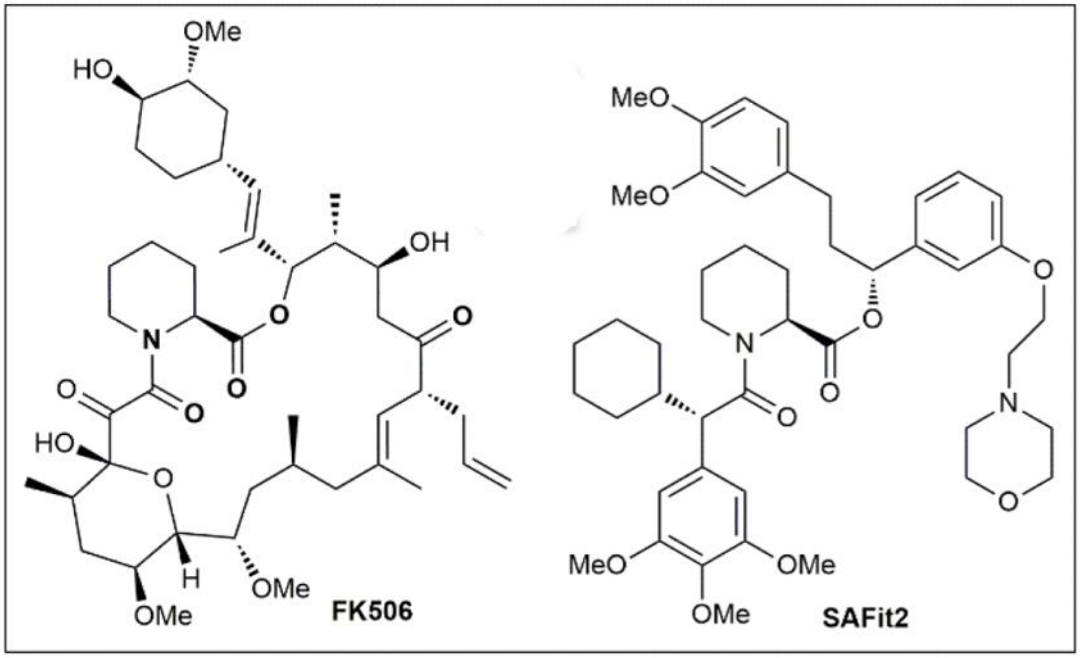

Figure 1.5. Chemical structure of FK506, the natural ligand for FKBP51, and of SAFit2. From Hahle et al. (349).

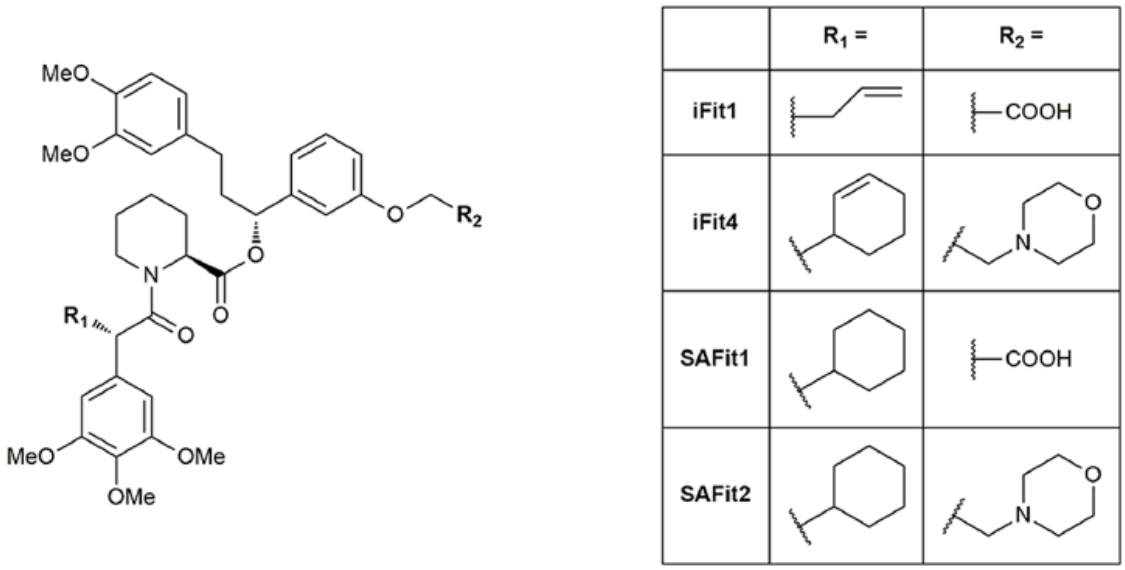

Figure 1.6. Chemical structures of iFit1 and iFit4, two early precursors in the SAFit development, and SAFit1 and SAFit2. Hahle et al. (349).

1.16. Pre-clinical studies using SAFit2

Early studies with SAFit2 in mice showed that a single i.p. injection with SAFit2 (20mg/kg) could reduce anxiety-like behaviour caused by FKBP51 overexpression in the BLA. The mice injected with SAFit2 spent more time in the open arms in the elevated plus maze test, more time in the lit compartment, and made more entries to the lit compartment in the light-dark box test, both indicating reduced anxiety (340). Furthermore, mice treated with SAFit2 demonstrated decreased CORT concentrations at the circadian peak, but not at the trough (347). Mice treated with SAFit2 and exposed to the DEX/CRH test showed enhanced suppression by DEX, which remained enhanced after stimulation with CRH as shown by decreased CORT concentrations (347). This suggests that the SAFit2 enhances the negative feedback mechanisms activated by DEX administration. Furthermore, SAFit2 had antidepressant properties in the forced-swim test, similar to those of commonly used antidepressants (347).

Another study investigated the effects of SAFit2 treatment on the efficacy of a highly prescribed selective serotonin reuptake inhibitor (SSRI), escitalopram, used for depression and anxiety treatment. The co-administration of SAFit2 reduced the efficacy of the SSRI, however, it increased the stress-coping behaviour in the mice. Mice were injected with an empty or SAFit2 loaded vesicular phospholipid gel (VPG), which allowed for prolonged treatment with SAFit2. Behavioural testing was performed on day 2 to 5 with an intraperitoneal (i.p.) injection of escitalopram or vehicle 30 minutes before the testing. In addition to behavioural data, the basal CORT and stress-induced CORT were also investigated. It was found that SAFit2 mitigated the anxiolytic effects of escitalopram in most of the behavioural tests performed. In contrast, SAFit2 increased struggling time in the forced swim test. When investigating the effects of SAFit2 on CORT concentrations it was found that SAFit2 when combined with escitalopram, had no effect on the stress-induced CORT however, it reduced the recovery time when compared to empty-VPG and escitalopram treated mice (350). The same group investigated the effect of SAFit2 on metabolism and its potential involvement in obesity. The study showed that 51KO mice are resistant to high fat diet-induced obesity. Furthermore, treatment with SAFit2 in mice mimics these effects on body weight and glucose tolerance. In addition, the SAFit2-mediated improvement in glucose tolerance preceded the decrease in body weight. The same study found that the mechanism

behind these effects involved the AS160, an AKT2 substrate. Treatment with SAFit2 increased the AS160 phosphorylation, which in turn increased the expression of the glucose transporter 4 at the plasma membrane, and hence improved glucose uptake in skeletal myotubes (351).

1.17. Rationale, Aims, and Hypotheses

Previous work involving FKBP51 in regulating the HPA axis has shown intriguing links between FKPB51 and CORT regulation. Studies performed in animals using SAFit2 have demonstrated a potential of FKBP51-inhibition to decrease HPA axis activity and ultimately CORT concentrations following a stressor. However, the studies investigating the effect of SAFit2 on basal HPA axis activity are limited and there are to date no studies investigating the effect of FKBP51-inhibition on the 24-hour pulsatile pattern of CORT release. In addition, SAFit1 has not been well characterised in regards to its effects *in vivo* and it has interesting potential as a peripheral-only FKBP51 inhibitor. Furthermore, the 51KO mice showed a stronger effect on basal HPA axis activity in the female mice compared to male mice, however, no studies using SAFit2 have been performed in females.

Because global knock-out of FKBP51 reduced both basal CORT concentrations (in females only) and stress-induced CORT concentrations in male and female mice, I was interested in investigating if similar results would be observable in rats. Because I did not have the availability of 51KO rats I decided to use two FKBP51-specific inhibitors, SAFit2 and SAFit1. The results from the 51KO mice studies, coupled with the results from studies in primates indicating that increased expression of FKBP51 causes GC resistance (321), and recent findings in humans (334), were all foundations to the rationale for my project.

The studies presented in this thesis were performed in rats, as more blood samples can be taken in a short period and frequent blood sampling and CORT concentration measured throughout 24 hours. The CORT concentrations were measured during 24-hours to establish whether the SAFit compounds affect the ultradian pulsatility of CORT secretion.

The overall aim of this research was to further characterise the involvement of GR and its chaperone, FKBP51. The general hypothesis was that by inhibiting FKBP51, GR activity

would be altered, and this would affect the GC negative feedback (fig 1.7.). The specific aims for the overall project were the following:

Aim 1: To elucidate the effects of SAFit2 and SAFit1 treatment on HPA axis activity in male rats.

Aim 2: To elucidate the effects of SAFit2 and SAFit1 treatment on HPA axis activity in female rats.

Aim 3: To elucidate the molecular mechanisms underlying the effects of SAFit2 and SAFit1 on HPA axis activity described in aim 1 and 2.

The objectives of the project were to elucidate these 3 aims by using an automated blood sampling system to measure CORT concentrations during 24 hours following both acute and sub-chronic (5 days) SAFit2 or SAFit1 treatment. To quantify the potential changes in CORT concentrations, and ultradian pulsatility, the PULSAR algorithm was used to analyse the pulsatile dynamics of CORT. Moreover, to characterise at what level of the HPA axis the SAFit1 and SAFit2 are active, the anterior pituitary gland and adrenal gland were stimulated with CRH, and ACTH, respectively. To elucidate the molecular mechanism behind any potential effects, subcellular translocation of GR following SAFit2 or SAFit1 was quantified.

The general hypothesis driving the rationale and aims for this project is that by inhibiting FKBP51, the GR will be activated and induce GC-mediated negative feedback, which ultimately will cause a reduction in CORT concentrations. More specifically, the hypothesis was that FKBP51 inhibition will cause GR to translocate to the nucleus and induce negative feedback regulation by inhibiting gene transcription, and/or secretion of CRH and POMC/ACTH, resulting in reduced CORT secretion from the adrenal glands. There is a long-standing notion that the CORT ultradian pulsatility is under hypothalamic regulation, however recent evidence suggests a role for a pituitary-adrenal gland feed-forward/feedback loop (132), though few studies have shown a change in pulsatility. It is feasible to hypothesise that inhibition of FBKP51 by the peripheral-only SAFit1 can influence the ultradian CORT pulsatility. Furthermore, because of the well-known sexual dimorphism in HPA axis activity, it is plausible that SAFit2 and SAFit1 will affect males and females differently, although the complexity of HPA axis regulation makes it difficult to hypothesise where or how this difference will occur.

Emerging evidence suggests a strong link between FKBP51 and psychiatric disorders with dysregulated HPA axis activity, such as depression (334). The FKBP51 is a promising target for novel therapeutics and further knowledge in the FKBP51-mediated regulation of HPA axis negative feedback will be of great importance for advancement in this area.

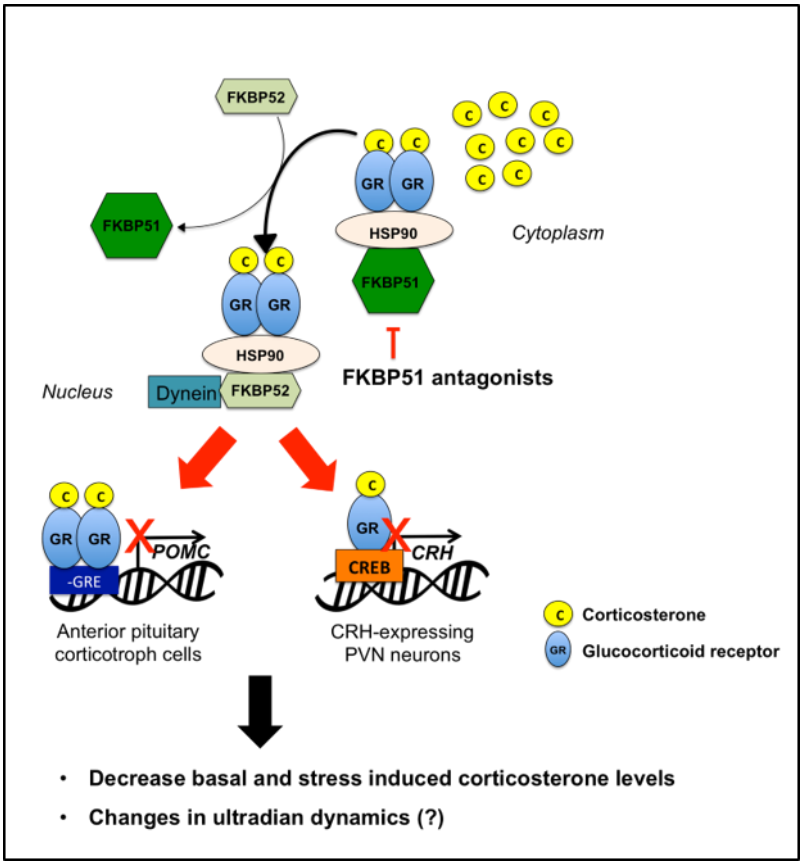

Figure 1.7. Hypothesis for the effects on HPA axis activity following FKBP51 antagonism. *FKBP51 and FKBP52 compete for binding to the GR and thus inhibition of FKBP51 would increase the binding of FKBP52 to the GR. This, in turn, will increase FKBP52 and dynein-mediated translocation of GR. Once in the nucleus, the GR can repress the transcription of HPA axis related genes such as CRH and POMC, which ultimately results in altered CORT secretion.*

Chapter 2: General methods

2.1. Animals

Male and female Sprague-Dawley rats were used in all experiments (except experiment 14) and were 7-8 weeks of age (190-224g) at the time of arrival in the animal facility. The rats were allowed to acclimatise to the facility and were handled once a day for one week before surgery or to the start of the experiment. Animals were maintained on 14 hours of light: 10 hours of darkness (lights on at 05.00h) except where otherwise indicated and had *ad libitum* access to food and water. The animals were group-housed, four per cage, except those subject to surgery, which were single-housed from one day before the surgery and during the experiment. The animals subjected to intracranial virus injection were singly housed for 5-7 days after the surgery, then regrouped until the surgery for jugular vein cannulation as described below. All experimental procedures were approved by the University of Bristol Animal Welfare Ethical Review Board and were conducted in accordance with the Home Office guidelines and the UK Animals (Scientific Procedures) Act, 1986.

2.2. Drugs

For the SAFit2 and SAFit1 experiments, the animals were treated via a subcutaneous (s.c.) cannula with either:

Vehicle: 4% EtOH, 5% Tween80 and 5% PEG300 in 0.9% sterile saline, SAFit2: 20mg/kg/ml, or SAFit1: 20mg/kg/ml.

For experiment 4, rats were injected in the intravenous (i.v.) cannula with 150ng CRH (1.5 µg/ml, Tocris, Bristol, UK) diluted in sterile saline.

For experiment 5, rats were injected in the i.v. with 40 ng ACTH (0.4 µg/ml, Synacthen, ACTH-(1–24) fragment; Alliance Pharmaceutical, Chippenham, UK) diluted in sterile saline.

For experiment 6, sterile saline was mixed with 2-hydroxypropyl-β-cyclodextrin (HBC) or CORT-HBC (0.3 mg/kg/ml- Sigma Aldrich, Missouri, USA) and administered as intraperitoneal (i.p) injections.

2.3. Experiments

The details of the experimental design of each study are outlined in the individual chapters. In brief, the following experiments were performed:

Experiment 1: Effect of acute SAFit2 treatment on CORT ultradian rhythm and stress-induced CORT secretion in male rats (chapter 3).

Experiment 2: Effect of acute SAFit1 treatment on CORT ultradian rhythm and stress-induced CORT secretion in male rats (chapter 3).

Experiment 3: Effect of acute SAFit1 and SAFit2 on basal ACTH concentrations in male rats (chapter 3).

Experiment 4: Effect of acute SAFit1 and SAFit2 on CRH-induced ACTH and CORT secretion in male rats (chapter 3).

Experiment 5: Effect of acute SAFit1 and SAFit2 on ACTH-induced CORT secretion in male rats (chapter 3).

Experiment 6: Effects of SAFit1 and SAFit2 on subcellular localisation and activation of GR and MR in the hippocampus and anterior pituitary gland in male rats (chapter 3).

Experiment 7: Effect of sub-chronic SAFit2 treatment on CORT ultradian rhythm and stress-induced CORT secretion in male rats (chapter 4).

Experiment 8: Effect of sub-chronic SAFit1 treatment on CORT ultradian rhythm and stress-induced CORT secretion in male rats (chapter 4).

Experiment 9: Effect of acute SAFit2 treatment on CORT ultradian rhythm and stress-induced CORT secretion in female rats (chapter 5).

Experiment 10: Effect of acute SAFit1 treatment on CORT ultradian rhythm and stress-induced CORT secretion in female rats (chapter 5).

Experiment 11: Effect of sub-chronic SAFit2 treatment on CORT ultradian rhythm and stress-induced CORT secretion in female rats (chapter 5).

Experiment 12: Expression of FKBP, GR, and MR in male and female brain and anterior pituitary gland (chapter 5).

Experiment 13: The effects of AAV-mediated overexpression of FKBP51 in the PVN on CORT ultradian rhythms and stress-induced CORT secretion in male rats (chapter 6).

Experiment 14: Characterisation of FKBP5 mRNA expression in the hypothalamus and anterior pituitary gland of Fischer and Lewis male rats (chapter 6).

2.4. Intravenous and subcutaneous cannulation surgery

Rats were handled individually for approximately 5 minutes *per* day for 5 days prior to surgery. The rats were fitted with a Covance Infusion Harness (Instech Laboratories Inc., Plymouth, PA, USA, see fig 2.1) on the day before surgery and were singly housed until the end of the experiment. This allowed the rats to acclimatise to the harness and single housing conditions. Rats were anaesthetised using isoflurane mixed with oxygen (1L/min): an induction chamber was used with 5% isoflurane until the rats were unconscious. Subsequently, the rats were moved to the shaving area and placed on a heating pad and kept under anaesthesia on 3% isoflurane. The rats were shaved on the ventral side between the head and the right front leg (approximately 2.5 x 2.5 cm) and the back between the shoulder blades after being wetted with 70% ethanol. A sharp scalpel and an electric shaver were used on the head and back, respectively. Once the rats had been shaved, they were moved to the surgical area and kept on 2.3-2.7% isoflurane throughout the surgery depending on the weight of the animal. During the shaving and surgery, the isoflurane was delivered using a fitted mask placed on the nose of the animal.

Once prepared for surgery the rats were placed on a heating pad covered in a sterile surgical drape. They were placed with the ventral side up, then covered in sterile plastic drape with a hole over the site of incision for the cannulation surgery. The skin at the site of incision was sterilised using chlorhexidine skin solution applied with a sterile cotton ball. Once the skin was sterilised, a scalpel was used to make an approx. 1.5 cm incision through the skin on top of the right jugular vein, lateral to the midline between the right front leg and the head, where the right jugular artery can be seen to pulsate.

Two tweezers were used to blunt dissect the underlying muscle and connective tissue to expose the right jugular vein, approx. 3 cm rostral to the opening of the right atrium of the

heart. Further, two smaller tweezers were used to isolate the right jugular vein and once isolated, a sterilised hairpin was pulled dorsally to the vein to secure it. Two 30 cm pieces of sterilised cotton thread were pulled underneath the hairpin and the rostral thread was tied around the vein to stop the blood flow from the head towards the site of the incision on the vein. The caudal thread was left loosely tied so that once the cannulation had occurred the caudal thread could quickly be securely tied around the cannula and the vein to stop any potential bleeding.

A thin ended tweezer was used to slightly lift the vein and small scissors were used to cut an approx. 1 mm incision in the vein, close to the rostral part of the hairpin. A pre-made cannula was custom cut to each animal, approx. 2.7 to 3.2 cm depending on the size of the animal and filled with heparinised isotonic sterile saline by attaching a saline-filled syringe connected with 0.5 ESCO tubing (Appleton Woods, Birmingham, UK) and a blunt pin. A small amount of saline was used to wet the vein and a thin tweezer was inserted into the incision to stretch it open so the tip of the cannula could be inserted. The cannula was then carefully inserted until the incision was at the point of the first lug (see fig 2.1.) and the end of the cannula was presumably at the opening of the right atrium. The saline-filled syringe was used to aspirate and make sure there was blood flushing through the cannula. Once the cannula was in place and flushing correctly, the cotton threads were tied and secured by making 3 knots, then the syringe and tubing were detached, and a bulldog clip was placed at the end of the cannula tubing to prevent blood leaking from the vein.

The next part of the surgery comprised exteriorisation of the cannula and the insertion of a subcutaneous cannula for drug administration. A sterile swab was placed on the open incision and the animal was moved to a supine position with the dorsal side facing up. The shaved area between the shoulder blades was sterilised using chlorhexidine applied with a cotton ball. A sterile 19G needle was used to pierce the skin at a point slightly lateral to the midline (to the right side of the rat) between the shoulder blades and a blunt needle attached to a saline-filled syringe was used to enlarge the hole. The same syringe and needle were used to make a narrow pocket directly underneath the skin from the incision between the shoulder blades to the incision of the cannulation. The blunt needle was cleared from any tissue and the cannula attached to it before passing it back through the small pocket underneath the skin. Once exteriorised on the back, the blunt needle and syringe were detached from the

cannula and the cannula was flushed using the flushing syringe. To insert the subcutaneous cannula, the 19G needle was used to pierce the skin at a point slightly lateral to the midline (to the left side of the rat) between the shoulder blades and a blunt needle attached to a saline-filled syringe was used to enlarge the hole. A small metal stick was used to make a subcutaneous pocket directly under the skin, long enough to fit the sub-cutaneous part of the cannula (8.5 cm – see fig 2.1). The cannula was inserted in the subcutaneous skin pocket and secured by a suture through the skin and wrapped between two lugs on the cannula, exterior to the skin.

The animal was put with the dorsal side up and the cannulation incision was sutured, and wound powder placed on the sutured incision. The isoflurane was turned down to setting 1 and finally, the intravenous and sub-cutaneous cannulas were attached to a metal stick and passed through a metal spring attached to the harness. The harness was pulled over the head of the rat and the two front legs passed through the side holes of the harness. Lastly, the animal was injected with an analgesic solution (Rimadyl, 200µl), the isoflurane was turned off and the animal was placed on a heated recovery area and monitored until fully awake. Once the animal was fully awake, it was transferred back in his cage and moved to the soundproof room where the automated blood sampling system is located. The rats were monitored regularly during recovery and kept on heated pads (on a timer: one hour on, one hour off) underneath the cages until the following morning.

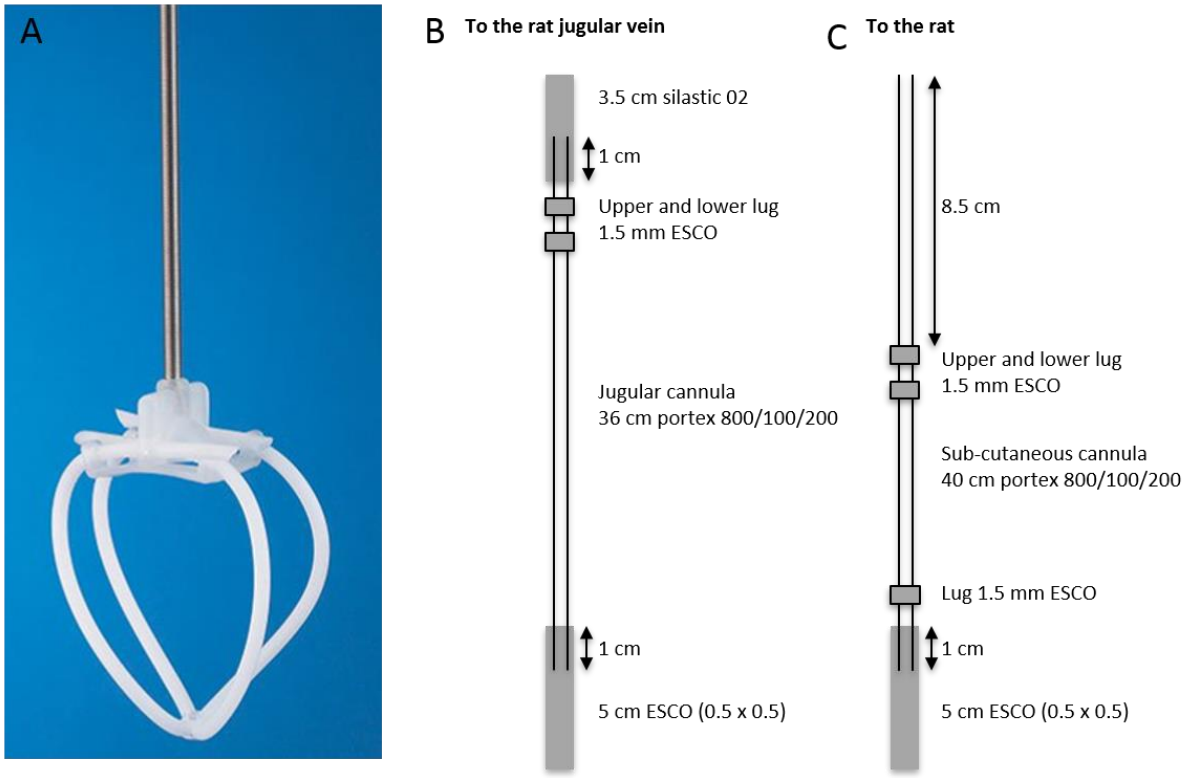

Figure 2.1. Covance Infusion Harness and cannula used for the jugular vein cannulation. *(A) The Covance Infusion harness in model CH105 was used to attach the metal spring in which the jugular vein cannula was passed through. (B) Schematic of the jugular vein cannula used, made from ESCO 0.5 x 0.5 (Appleton Woods, Birmingham, UK), silastic 02 tubing (Appleton Woods, Birmingham, UK), and portex 800/100/200 (Smith Medicals, Ashford, UK). (C) Schematic of the subcutaneous cannula used for SAFit2 and SAFit1 injections. The subcutaneous cannula was made from ESCO 0.5 x 0.5, and portex 800/100/200.*

2.5. Adrenalectomy

In experiment 6, to remove endogenous corticosterone, and ensure that similar levels of plasma corticosterone were achieved after intraperitoneal injection of preformed water-soluble corticosterone 2-hydroxypropyl-β-cyclodextrin (HBC), all rats were bilaterally adrenalectomized. The anaesthetized rat was placed ventral side down on a warm pad. The area on the mid-dorsum was shaved and chlorhexidine skin solution applied to the area with a sterile cotton ball. A midline incision of approximately 2 cm long was made just caudal to the peak of the animal's dorsal hump. Blunt-tipped scissors were inserted subcutaneously

through the incision and used to bluntly dissect the connective tissue, muscle, and peritoneum in both sides at approximately 2 cm below the last rib. Curved forceps were inserted through the incision into the peritoneal cavity to help locate each gland, then using the same forceps each adrenal gland was manipulated by grasping peri-adrenal fat and exteriorized taking good care to not to grasp the gland itself, because tearing of the gland may result in leaving functional residual tissue within the abdomen. The skin incision was then sutured, and powder applied. After the surgery, rats were subcutaneously injected with a solution of glucose in 0.9% saline and with an analgesic solution (Rimadyl, 200 µl). After recovery, drinking water was replaced with corticosterone (15 µg/ml) dissolved in 0.1% ethanol in 0.9% saline. Twelve hours before the onset of the experiment the rats were given 0.9% saline solution as drinking water.

2.6. Automated blood sampling

To allow for frequent, and stress-free blood samples to be collected from the rats, an automated blood sampling (ABS) system was used (93, 106, 352). This system has been used previously as a reliable blood sampling apparatus (106, 239, 240, 352, 353). The cannula collects blood from the rat with the help of a peristaltic pump which drives the collection. The blood is collected via the swivel and a 3-way valve to the reservoir (10 cm portex 800/100/580/100). The 3-way valve is connected to the cannula, the pump line which contains the reservoir, and the fraction line. The fraction line collects the 37.7 µl for each blood sample from the reservoir and via the fraction line is collected in microtiter tubes in the fraction collector (fig 2.2 -2.4.)

The pump line is made from portex 800/100/200, black/black tube, ESCO 0.5 x 0.5 mm, and silastic 03 (Smiths Medical Inc, Minneapolis, MN, US). The fraction line is made from portex 800/100/100, fraction head tubing, silastic 01, ESCO 0.5 x 0.5 mm (Smiths Medical Inc, Minneapolis, MN, US), and the needle used is a 22G or 23G blunt needle. The cannula implanted in the rat is described above.

The ABS was programmed to collect blood samples every 10 minutes for 25 hours starting at 07.00h. Each blood sample contained 37.7 µl blood and was diluted in a ratio of 1:5 in heparinised saline.

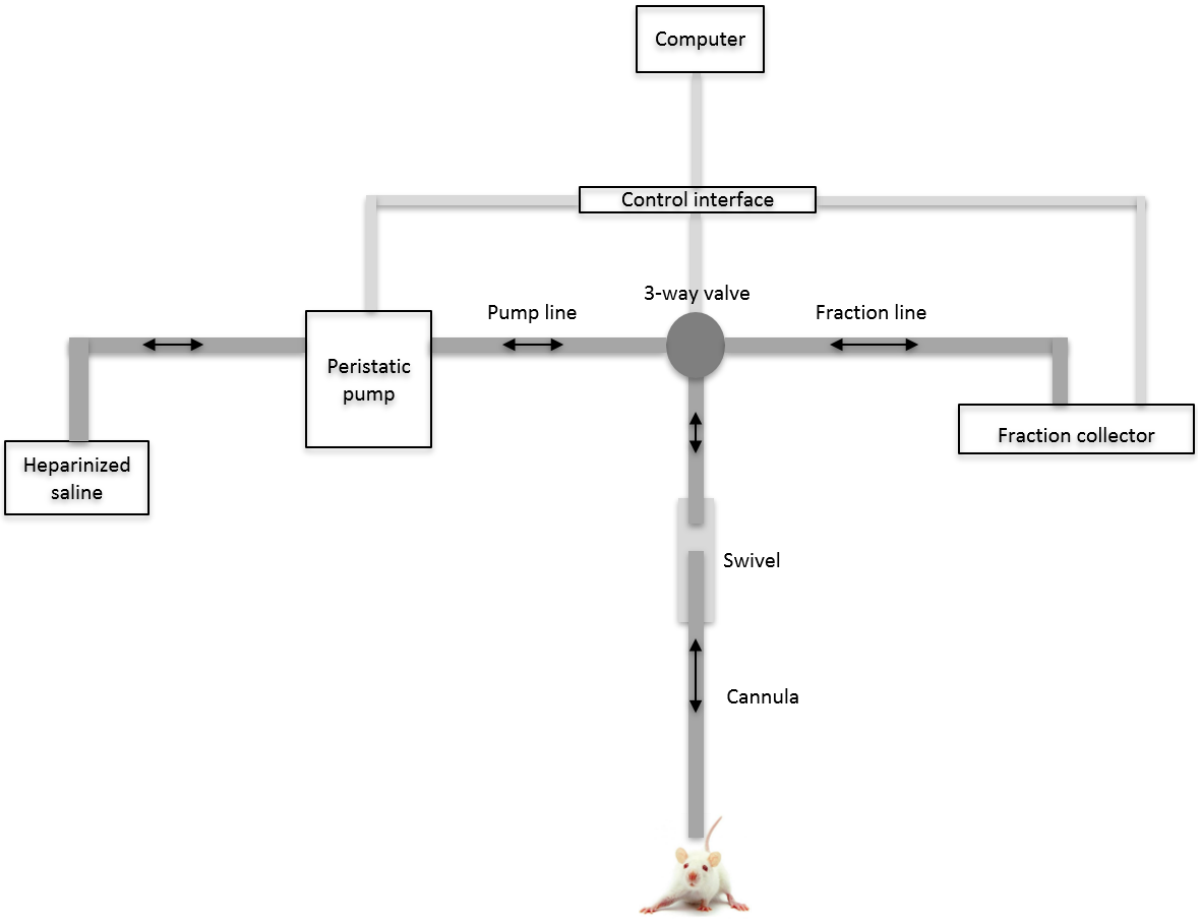

Figure 2.2. Overview of the automated blood sampling system (ABS). *The ABS allows for frequent blood sampling and is controlled remotely to avoid interference with the animals. The blood samples are collected via a cannula implanted in the right jugular vein in the rat.*

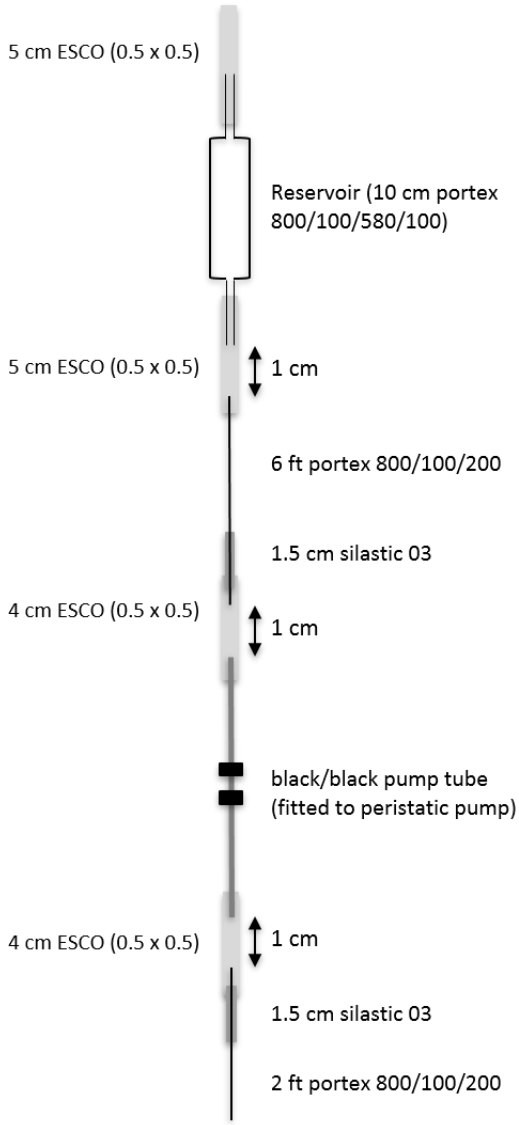

Figure 2.3. Specifications for the pump line. *The reservoir holds a larger amount of the blood for the collection of the exact 37.7 µl via the fraction line by switching the 3-way valve. The rest of the blood in the reservoir is pumped back in the rat by switching the valve to the cannula.*

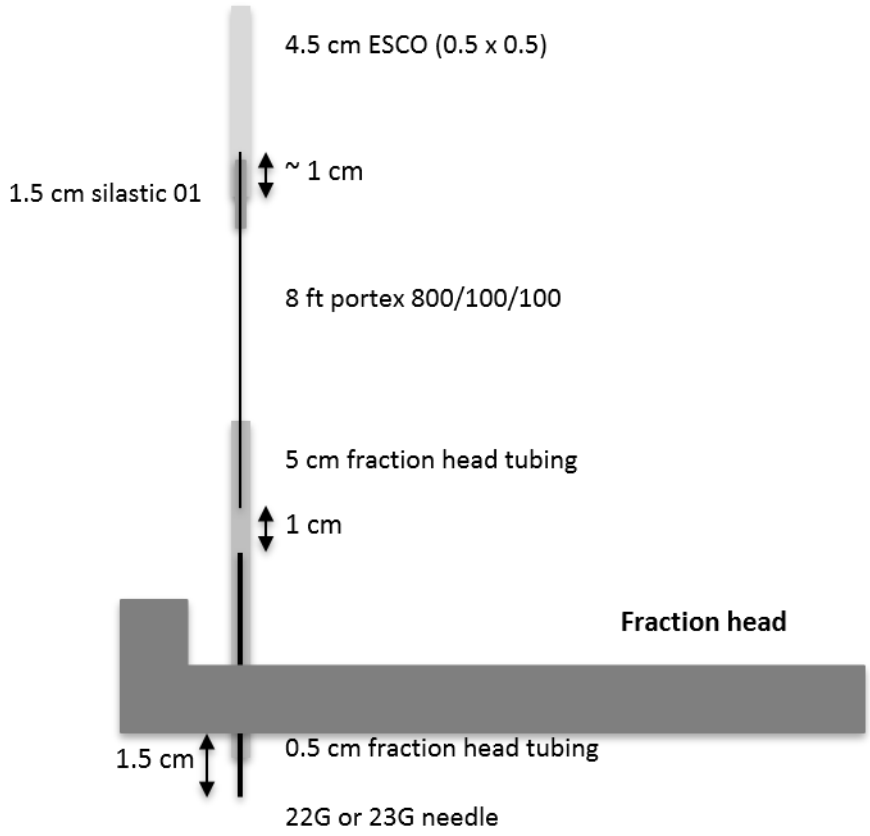

Figure 2.4. Specifications for the fraction head*. The blood is collected via the cannula to a reservoir connected to a 3-way valve. To collect the blood samples in the microtiter tubes, the fraction line collects blood from the reservoir and deposit in the tubes via the 22G or 23G needle attached to the fraction head. The fraction head moves along two axes to allow for the precise deposit of the blood in the correct tubes.*

2.7. Manual blood sampling

From each rat, blood samples were taken by hand using a cannula implanted in the right jugular vein (see surgery section 2.4). The blood samples were taken using a blunt needle attached to a 1 ml syringe. A flushing syringe comprising a 30cm ESCO 0.5 x 0. 5mm tubing attached to a blunt needle and 1 ml syringe was used before the blood sample to aspirate blood through the cannula. Approximately 200 µl blood was taken from each rat at each time point and to maintain blood volume, each blood sample was replaced by 200 µl of heparinised saline. The blood was deposited in a 1.5 ml Eppendorf tube, containing 10 µl EDTA to prevent coagulation, and immediately mixed for a few seconds. The samples were then centrifuged

at 4°C at 4000 rpm for 15 minutes to separate the plasma. The plasma was aliquoted in two tubes; one for CORT measurements (~10 µl) and one for ACTH measurements (~110 µl), then snap-frozen on dry ice, and stored at -80°C until processing for ACTH and CORT measurements.

2.8. Corticosterone measurement

The CORT concentrations in unextracted blood or plasma samples were measured by radioimmunoassay (RIA). A specific rabbit polyclonal anti-corticosterone antibody developed by Professor Gabor Makara (Institute of Experimental Medicine, Budapest, Hungary)(354), and kindly donated to us by Dr D'ora Zelena (Institute of Experimental Medicine, Budapest, Hungary) was used at the final dilution of 1:40000. I^{125}–labelled corticosterone was used as a tracer (Institute of Isotopes, Budapest, Hungary) and dissolved in 1 ml of distilled water and then further diluted in a citrate buffer (14.6 g trisodium citrate, Sigma-Aldrich; 13.8 g sodium dihydrogen orthophosphate, Sigma-Aldrich; 2 g bovine serum albumin (BSA), Sigma-Aldrich; 2 l purified water; hydrochloric acid to pH 3.0) to achieve an activity of 3500-4000 CPM in 50 µl of tracer solution. To measure CORT in blood samples obtained from male rats using the ABS, 50 µl of the blood-heparin saline mix was further diluted in 50 µl of citrate buffer. The use of a low pH citrate buffer allows denaturation of plasma corticosteroid-binding globulin (CBG), which can interfere with the assay. Dilution of samples obtained from female rats was 20 µl blood in 80 µl citrate buffer to avoid female CORT levels to fall outside the corticosterone standard curve range (0.1-100 ng/ml). For the hand samples, 5 µl of plasma was diluted in 495 µl citrate buffer and processed in triplicates. The samples were incubated with 50µl of corticosterone tracer and 50 µl antibody at 4°C overnight. The following day 500 µl charcoal-dextran was added to each sample, the samples were mixed thoroughly and immediately centrifuged (4000 RPM, 4°C for 15 min). The precipitant solution allows the I^{125}-corticosterone that did not bind to the antibody to be captured and precipitate. The liquid phase from each sample was then aspirated using a fixed water pump and each tube containing the charcoal pellet was loaded and counted using a Wizard II Gamma Counter (Perkin Elmer, MA, US). The quality of the CORT RIA was verified by using internal controls of known CORT concentration (20 ng/ml; 100 ng/ml). Levels of non-specific binding were assessed by comparing counts from

"totals" (i.e. tracer only) and "blanks" (i.e. tracer and charcoal). A standard curve (figure 2.5) was used to calculate the CORT concentrations in the samples. The intra- and inter-assay coefficients of variation of the corticosterone RIA were 14.1% and 15.3 %, respectively. The lowest detectable limit was 0.1 ng/ml.

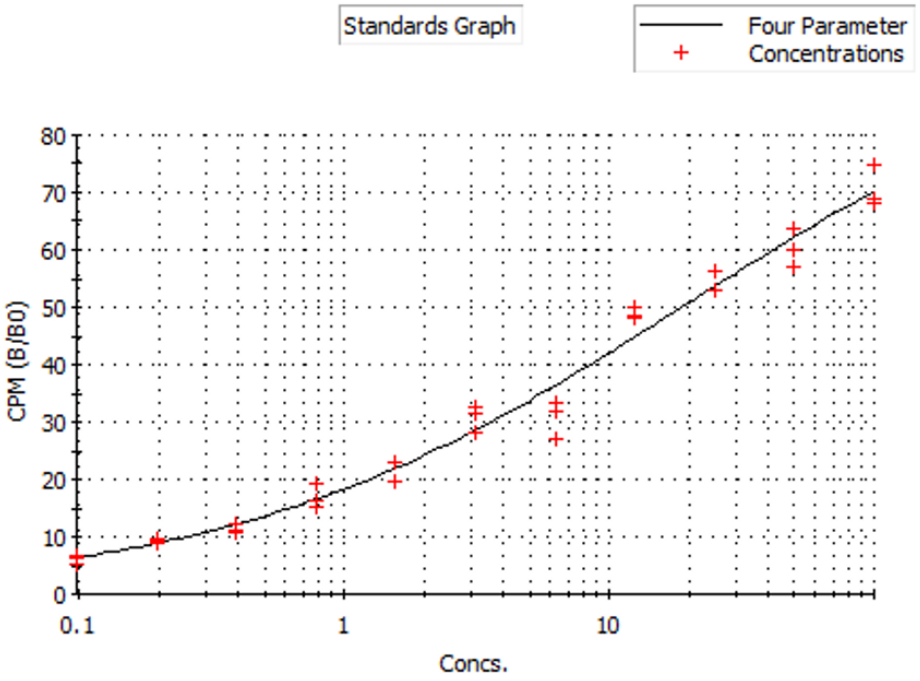

Figure 2.5. CORT RIA standard curve. *A representative of the standard curve from one of the CORT radioimmunoassay performed as previously described. The CORT concentrations (ng/ml) for each standard are plotted against the corrected CPM (%). Each standard comprised of three replicates.*

2.9. ACTH measurement

For the ACTH measurement, the 0.2 ml blood samples from the hand sampling procedures were collected in ice-cold tubes containing 10 µl EDTA (0.5 M; pH 7.4) and 10 µl aprotinin (500,000 KIU/ml, Trasylol; Bayer, Newbury, UK). The tubes were immediately centrifuged (4000 RPM, 4°C for 15 min) to separate the plasma, and the plasma was stored at -80°C until processed. ACTH concentrations were measured in single samples of 100µl plasma,

using ImmuChem Double Antibody 125I RIA Kit (MP Biomedicals, Orangeburg, NY, USA) in accordance with the manufacturer's protocol. One measurement per sample was used due to the low volume of blood obtained from the sampling.

The samples were incubated with 100 µl of ACTH 125I tracer and 100 µl antibody at 4°C overnight. The following day 500 µl precipitate solution was added to each sample, the samples were mixed through and immediately centrifuged (4000 RPM, 4°C for 15 min). The liquid phase from each sample was aspirated using a fixed water pump and each tube containing the precipitated pellet was loaded and counted using a Wizard II Gamma Counter (Perkin Elmer, Waltham, MA, US). The quality of the ACTH RIA was verified by using two internal controls of known ACTH concentration (which varies between ACTH kits). The concentrations of the samples were calculated using a standard curve (figure 2.6). The intra- and inter-assay coefficients of variation of the ACTH RIA were 6.8% and 10.7 %, respectively. The lowest detectable limit according to the manufacturer is approximately 5.7 pg/ml.

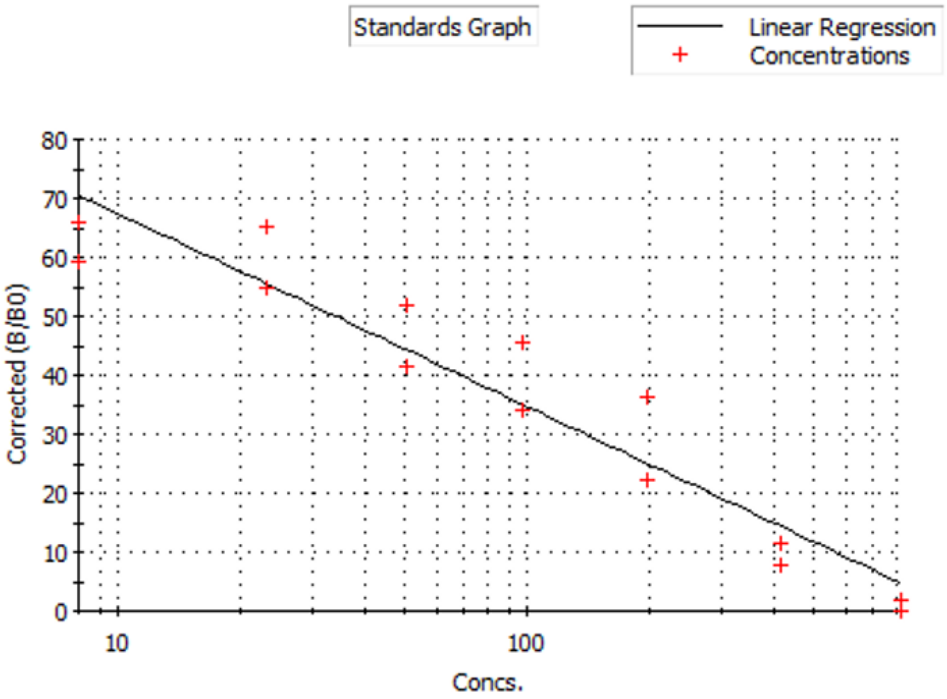

Figure 2.6. ACTH RIA standard curve. *A representative of the standard curve from one of the ACTH radioimmunoassay performed using the ImmuChem Double Antibody 125I RIA Kit (MP Biomedicals, Orangeburg, NY, USA) as previously described. Three replicates were used for* each standard and the ACTH concentrations (pg/ml) are plotted against the corrected CPM (%).

2.10. Tissue collection

At the end of experiments 1, 2, 6-12 and 14, the brain and pituitary gland tissue was collected for examination of the effects of SAFit1 and SAFit2 on the expression of genes of interest. Rats were rapidly euthanized with either isoflurane (less than 15 seconds exposure; experiment 6 and 12 only) or pentobarbitone (all other experiments; 0.2 ml, I.V., Merial, UK) followed by decapitation with a guillotine. After decapitation, the skull was opened using scissors, the brain excised and the hypothalamus, prefrontal cortex, and hippocampus dissected out. The pituitary gland was excised, and the intermediate and posterior lobe removed. All areas were snap-frozen on dry ice and stored at -80°C until processed for real-time quantitative polymerase chain reaction (RT-qPCR) or Western immunoblotting.

Work from our group and others has shown that this technique used for euthanising rats does not induce an increase in ACTH or CORT secretion (133, 355).

2.10.1. Anatomical boundaries for brain dissection

Prefrontal cortex: the brain was placed with the ventral side facing the metal plate and using a sharp razor blade the olfactory bulb was removed and an approximately 5 mm thick slice containing the prefrontal cortex was cut (figure 2.7).

Hypothalamus: the brain was placed with the ventral side facing up and using curved fine forceps the area between the optic nerve chiasm and the mamillary body was dissected (figure 2.7).

Hippocampus: the brain was placed with the ventral side facing the metal plate and using a sharp razor blade the two cerebral hemispheres were separated. From each hemisphere, the cortex was lifted to expose the hippocampus that was gently rolled out using curved forceps (figure 2.7).

Anterior pituitary gland: after collection, the pituitary gland was placed with the posterior lobe facing up and using a sharp blade the two lobes at each side of the intermediate lobe were dissected (figure 2.7).

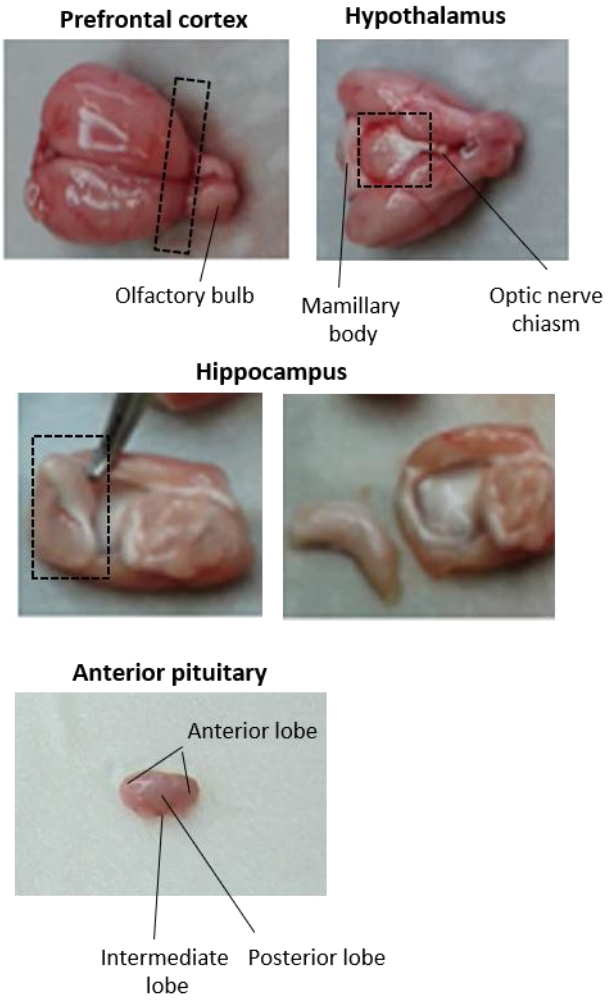

Figure 2.7. Anatomical boundaries for brain dissection. *Representative images of the dissection of the prefrontal cortex, hypothalamus, hippocampus, and the anterior pituitary gland from the rat.*

2.11. RNA extraction and Reverse Transcript

TRIzol (Invitrogen, Hopkinton, MA, USA) was used to isolate RNA from the hippocampus (experiment 12 only), the pre-frontal cortex (experiment 12 only), hypothalamus and anterior pituitary gland. RNA purification and removal of genomic DNA were performed using RNeasy mini kit reagents and column DNase digestion (Qiagen Valencia, CA, USA) respectively, as described by the manufacturer.

The RNA concentration and quality were measured using a NanoDrop Microvolume Spectrophotometer and Fluorometer (Thermo Fischer Scientific). Complementary DNA was

obtained by reverse transcription with Cloned AMV First-Strand cDNA synthesis kit (Invitrogen, Hopkinton, MA, USA) from 1 µg of total RNA. For each sample, 1µg of RNA was diluted in 11 µl RNase-free water and 1µl deoxynucleotide triphosphate (dNTP) mix, and 1 µl random primers (both provided by Invitrogen), and the samples were mixed for 5 seconds. The samples were centrifuged briefly and incubated for 5 minutes at 65°C using a PCR system 9700 Fast Thermal Cycler (GeneAmp, Thermo Fischer Scientific). Following the incubation, 4µl of provided 5x buffer, 1 µl 0.1M dithiothreitol (DTT), 1 µl RNase OUT, and 1 µl Cloned RT was added to each sample. The samples were vortexed and centrifuged briefly and then incubated for 10 minutes at 25°C, 50 minutes at 50°C, and 15 minutes at 70°C in the same PCR thermal cycler.

Genomic DNA contamination was assessed in 1 to 3 samples/reaction set by omitting the reverse transcriptase enzyme from the reaction mix. The absence of amplification products was then verified by RT-qPCR as described below, indicating the absence of genomic DNA contamination.

2.12. Real-Time qPCR

In each experiment a 1:10 dilution of stock cDNA was made to achieve a working concentration of cDNA of 1 ng/µl; 2 µl per sample of cDNA was used in duplicates. Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and forward and reverse primers at a final concentration of 200 nm were added to the amplification mixture to make up the final reaction volume of 25 µl. A spectrofluorometric thermal cycler (7500 Real-Time PCR System; Applied Biosystems) was used to perform the PCR reactions, comprising an initial cycle at 50°C for 2 minutes, 95°C for 10 minutes and subsequently 40 to 45 cycles at 95°C for 20 seconds, and 60°C for 1 minute. Primers were designed using Primer Blast (NIH, US National Library of Medicine) to span across exon-exon to amplify specifically cDNA corresponding to the mRNA of target genes (table 2.1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression was determined and used as the house-keeping gene to normalise all target gene expression levels. Glyceraldehyde 3-phosphate was used because of its previously determined stable expression in the tissues investigated.

Table 2.1. *List of primers used for RT-qPCR*

Gene	Forward primer	Reverse primer
AVP	TGCCTGCTACTTCCAGAACTGC	AGGGGAGACACTGTCTCAGCTC
cFos	AGCATGGGCTCCCCTGTCA	GAGACCAGAGTGGGCTGCA
CRH	CTCTCTGGATCTCACCTTCCAC	CTAAATGCAGAATCGTTTTGGC
FKBP5	GAACCCAATGCTGAGCTTATG	ATGTACTTGCCTCCCTTGAAG
GAPDH	CCATCACTGCCACCCAGAAGA	GACACATTGGGGGTAGGAACA
GILZ	CCATGGATCTAGTGAAGAATCATTTG	CCACCTCCTCTCTCACAGCATAC
GR	CACAGCTCACCCCTACCTTG	TTTCTGAAGCCTGGTATCGCC
MR	CAAGGACCATCGGTGAACT	TTGGTCCTCGAGAGGCAAGT
PER1	CCTCGATGTAACGGCTTGTGT	GTCCGAGTGGCCAGGATCT
POMC	AGAGCTGGCTTTCCGCGACA	GGGCCCCTGAGCGACTGTAG
SGK1	CGTACGACCGGACAGTGGA	GATATTTGGTTTCAGCTGGAGAGG

2.13. Subcellular fractionation

Cytoplasmic, membrane, nuclear and chromatin extracts were obtained using an extraction kit (Subcellular Protein Fractionation Kit, Thermo Fisher Scientific Inc., Rockford, IL, USA) according to the manufacturer's protocol. Thermo Scientific™ Halt™ Protease Inhibitor Cocktail was added at 1:100 dilution to the Cytoplasmic extraction buffer (CEB), membrane extraction buffer (MEB), and nuclear extraction buffer (NEB), and the buffers were kept on ice. Each extraction buffer contains detergents to extract the proteins sequentially (figure 2.8.).

The hippocampus and anterior pituitary gland were homogenised in 1000 µl (hippocampus) or 200 µl (anterior pituitary gland) CEB using a Dounce homogeniser and were vortexed for 5 seconds and then centrifuged (3000 RCF for 5 minutes). The supernatant (soluble cytoplasmic extract) was transferred to a fresh, pre-chilled tube. Further, 650 µl (hippocampus) and 100 µl (anterior pituitary gland) MEB was added to the pellet and the tubes were vortexed and then incubated for 10 minutes on ice with occasional mixing, before being centrifuged (3000 RCF for 5 minutes). The supernatant (soluble membrane-bound extract) was transferred to new pre-chilled tubes. The NEB (225 µl for the hippocampus, 40 µl for the anterior pituitary gland) was added to the pellet, the tubes vortexed and then incubated on ice for 30 minutes with occasional mixing. The tubes were centrifuged (5000 RCF for 5 minutes) and the supernatant (soluble nuclear extract) transferred to a new, pre-chilled tube. The chromatin buffer (CB) was made by adding 5 µl of CaCl2 and 3 µl of MN to

100 µl of NEB, and 170 µl (hippocampus) and 40 µl (anterior pituitary gland) of CB was added to the pellet. The tubes were vortexed for 15 seconds. The tubes were then incubated at room temp for 30 mins or in 37°C water bath for 15 mins followed by mixing for 15 seconds and centrifuged (16000 RCF for 5 minutes), and the supernatant was collected (soluble chromatin-bound extract).

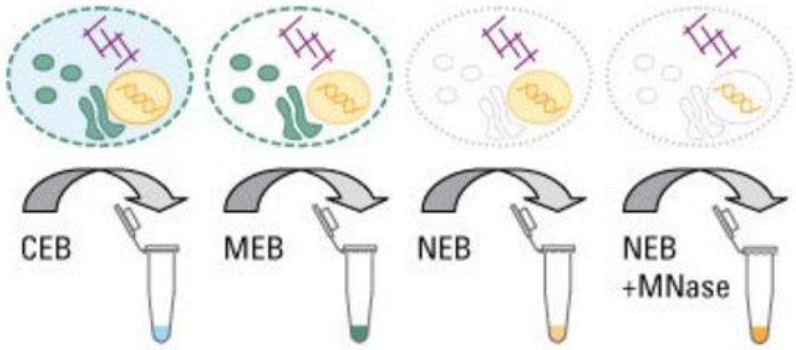

Figure 2.8. Schematic of the subcellular fractionation protocol. *Extracted using differential detergents. CEB = cytoplasmic extraction buffer; MEB = membrane extraction buffer; NEB = nuclear extraction buffer; NEB + MNase = nuclear extraction buffer plus micrococcal nuclease (chromatin-bound fraction).*

The protein concentrations for all extracts were measured by spectrophotometry by using a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). The cytoplasmic fractions (and the nuclear fractions for hippocampus) were measured in a 1:10 dilution with cytoplasmic extract, and the membrane, nuclear, and chromatin fractions were measured in a 1:2 dilution in the respective extract. Following dilution, each sample was loaded in duplicates on a 96-well plate. Standards with known concentrations of 0.05 to 1.0 mg/ml were loaded in duplicates to create a standard curve. The BCA mixture was prepared by mixing Protein buffer B with Protein buffer A in a 1:50 dilution, and 200 µl was added to each well, containing the samples of unknown concentrations and the standards. The plate was covered with aluminium foil and incubates at 37°C for approximately 40 minutes. An iMARK microplate reader (BioRad) was used to measure the absorbance in the wells at 570 nm wavelength.

2.14. Western immunoblotting

Samples from each region were run on a 4-15% Tris-glycine gel (Bio-Rad, Hercules, CA, US). Sodium dodecyl sulphate (SDS) buffer was used to make the final protein concentration of either 1 µg/µl (for the anterior pituitary gland) or 2 µg/µl (for the hippocampus). 10 µl of each sample was loaded and run on premade gels (BioRad) for approximately 1 hour on 35mA (2 gels), or 70 mA (4 gels). The samples were then transferred onto a polyvinylidene fluoride (PVDF) membrane (GE Amersham Biosciences, Piscataway, NJ, US) for 90 minutes at 250 mA.

The transferred membranes were then blocked using 1% BSA (Sigma) or 5% milk in 1x Tris buffer saline with 0.05% Tween 20 (TBST). Membranes were then probed overnight with primary antibodies (table 2.2), washed with TBST and then probed with secondary antibody (anti-rabbit or anti-mouse, all at 1:10000 dilution) for 1 h at room temperature. After further washes in TBST, Luminata Forte Western HRP Substrate (Millipore Corporation, Billerica, MA, US) was used for chemiluminescence and developed using G-Box (Syngene Int. Ltd., Bangalore, India).

The proteins were quantified using ImageJ to determine the relative optical density of each band for the protein of interest, which was then normalised to the background optical density.

Table 2.2. *Primary and secondary antibodies for Western immunoblotting. The primary and secondary antibody dilutions were made in TBST and incubated overnight at 4°C.*

Antibody	Dilution	Company
Rabbit anti-pCREB	1:1000 in 1% BSA	Cell Signaling Technology, Inc., Danvers, MA, USA
Rabbit anti-GR (M-20)	1:1000 in 0.1% milk	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA
Rabbit anti-pGR	1:1000 in 1% BSA	Cell Signaling Technology, Inc., Danvers, MA, USA
Rabbit anti-lamin	1:1000 in 1% BSA	Cell Signaling Technology, Inc., Danvers, MA, USA
Rabbit anti-H1	1:1000 in 1% BSA	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA
Rabbit anti-GAPDH	1:1000 in TBST	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA
Rabbit anti-Pan-cadherin	1:1000 in TBST	Cell Signaling Technology, Inc., Danvers, MA, USA
Rabbit anti-MR (H-300)	1:1000 in 5% milk	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA
Horseradish peroxidase-conjugated donkey anti-rabbit	1:10 000 in 1% BSA or 5% milk	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA
Horseradish peroxidase-conjugated donkey anti-mouse	1:10 000 in 1% BSA or 5% milk	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA

2.15. PULSAR analysis

The PULSAR algorithm (356) was used for comparison of pulse parameters from the ABS experiments. The PULSAR algorithm is based on the parameters of the specific hormone measured (e.g. plasma half-life), detail of the measurement assay (minimum detectable concentration) the performance of the hormone assay, and detail of the experimental design (e.g. frequency of sampling, duration of the experiment, number of samples). The measurement assay performance (i.e. the standard deviation, SD) is calculated for the assay used and the estimated SD-values can then be fitted to a quadratic regression curve from which the parameters A, B, and C can be calculated. For example, if the B=10, and the A=0, and B=0, this tells us that the coefficient of variation (CV) is 10%. These parameters must be calculated for each specific assay performed. I used the triplicates of the CORT RIA standards to calculate the SD for the respective standard concentrating. A curve was fitted to the values to obtain the A, B, and C values which in my setting were: A=0.035, B=2.63, C=6.15 (figure 2.9.). When the hormone data are analysed using PULSAR, a baseline is calculated to which the pulsatile data is compared. If a single time point has a higher value (in SD-units) than the G_1 value, then that time point is considered a peak (pulse). Two adjoining time points which both have higher values than the G_2 value are considered a peak, and so on.

The following G-values were employed: G_1=5, G_2=3, G_3=2, G_4=1.5, and G_5=0.8 together with a peak splitting parameter of 3 (S.D. units). The splitting parameter indicates how many SD units a trough must consist of in order to split a peak into two. These values were obtained from visual inspection of the data, as recommended (105).

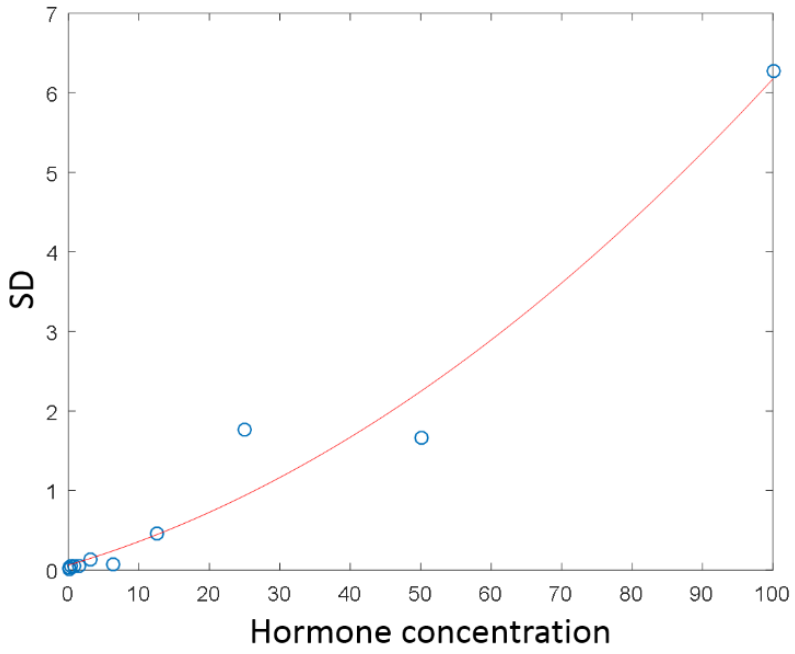

Figure 2.9. Generation of A, B, and C parameters for the PULSAR algorithm. *The mean SD values from CORT RIA standard triplicates for respective concentrations. A curve was fitted to the values and used to calculate the A, B, and C values for PULSAR.*

The PULSAR algorithm can quantify the following parameters: mean CORT (the average concentrations of CORT during the 24-hours measurements), maximum CORT (the highest concentrations of CORT during the 24-hours measurements, baseline CORT (the average concentrations of CORT at the troughs of each pulse during the 24-hours measurements), number of pulses, pulse amplitude, pulse height, pulse length, pulse area, inter-pulse interval (IPI), area under the curve (AUC) basal, and AUC total. Some of them are explained below (figure 2.10.). The AUC basal is the area under the curve calculated from the baseline, whereas the AUC total is the total area under the curve. Finally, to quantify the frequency the number of pulses was divided by 24, as the sampling continued for 24 hours.

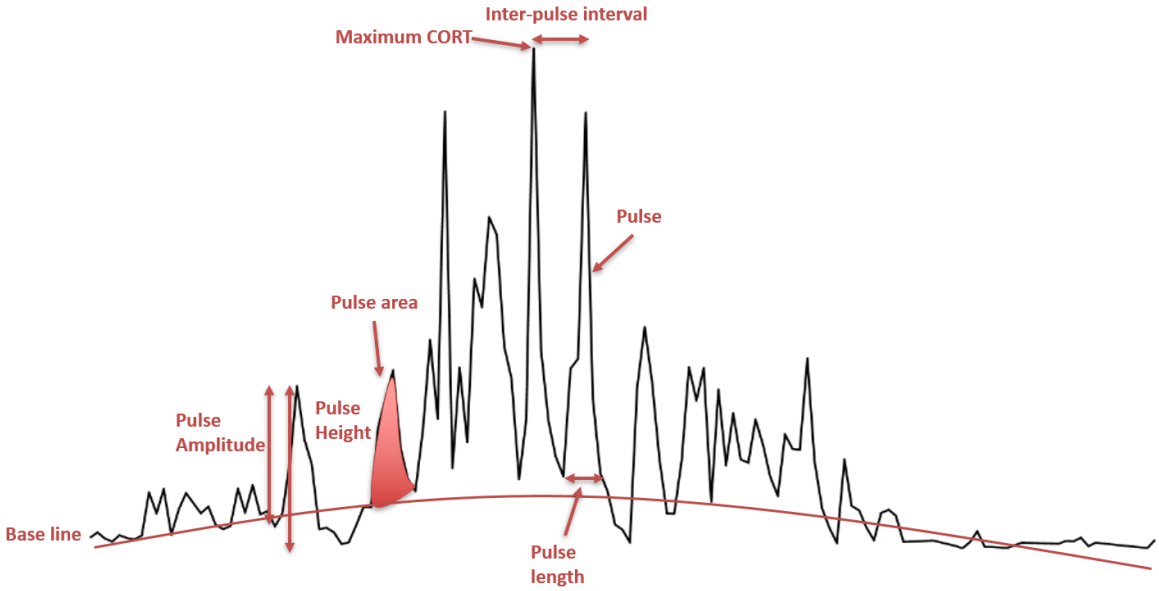

Figure 2.10. The parameters quantified by PULSAR algorithm. *The baseline is an estimated line that should go along the base of each pulse.*

2.16. Statistics

All statistical analyses were performed using SPSS version 11.5 for Windows (SPSS Inc., 12 Chicago, IL). Differences between groups were analysed by Tukey's post hoc test, or Fisher's least significant difference (LSD) post hoc test (experiment 4 and 5 only). Statistical significance was set at $P < 0.05$.

Chapter 3: Acute treatment with FKBP51 inhibitors in male rats

3.1. Introduction

In this chapter data on the effects of acute SAFit1 and SAFit2 treatment on the HPA axis activity in male rats will be presented. As explained in the introduction, SAFit1 and SAFit2 are FKBP51 inhibitors that differ in a few aspects. Firstly, SAFit1 does not penetrate the blood-brain barrier (BBB), hence it is only peripherally active; furthermore, it has a shorter half-life compared to SAFit2 (SAFit1 half-life: 2.5 hours, SAFit2 half-life: 9.7 hours). These differences might, therefore, contribute to differential effects between the two compounds. By inhibiting FKBP51 it would be expected to observe an increase in GR translocation into the nucleus, and subsequent increase or decrease in GR-regulated gene transcription, depending on whether GR activity is enhancing or inhibiting gene transcription (e.g. if GR bind to a GRE or an nGRE). The GR is involved in fast negative feedback, both in the brain and the pituitary gland (148). Therefore, the effects of acute treatment with SAFit1 and SAFit2 on basal CORT secretion were investigated. The hypothesis is that FKBP51 inhibition will enhance GR binding to the nGRE in the POMC gene in corticotrophs of the anterior pituitary gland or bind to CREB in CRH producing cells of the PVN, resulting in reduced transcription of the POMC and the CRH genes, and subsequently reduced CORT plasma levels. Many studies have demonstrated central regulation of the stress-induced CORT negative feedback, however, the stress response is also regulated by fast negative feedback occurring at the level of the pituitary gland (357). Therefore, whether inhibition of FKBP51 had any effect on stress-induced CORT secretion and whether these effects were different following treatment with SAFit1 or SAFit2 were also investigated.

Previous studies using male FKBP51 knockout mice (51KO) revealed no major differences in basal behaviour or neuroendocrine effects compared to wild-type littermates, although a dampened stress response was seen in the 51KO mice (358). This led to the interest in characterizing the effects of SAFit2 in male rats. Further, the effects of the peripheral-only FKBP51 inhibitor SAFit1 have not been characterised in mice nor rats and rendered a novel opportunity to investigate the effects of SAFit1. Although previous studies with SAFit2 and FKBP51 knockouts were all done in mice, to be able to study ultradian

rhythmicity of CORT secretion, rats were used in the studies presented in this thesis. The rat is larger and therefore frequent blood sampling can be performed to obtain basal, 24-h CORT profiles in unstressed freely moving rats. Moreover, manual sampling procedure can be done in the rat to obtain larger volumes of blood (~ 200 µl) without the stress of the handling procedure, to allow for measurement of ACTH concentrations.

3.2. Methods and experimental design

In all the studies reported in this chapter, except for experiment 6, male Sprague-Dawley rats were subject to the jugular vein and subcutaneous cannulation (see section 2.4 in the methods) and allowed to recover for at least 5 days before the start of the experiment, unless stated otherwise.

3.2.1. Experiments 1 and 2: Effect of acute SAFit2 and SAFit1 treatment on CORT ultradian rhythm and stress-induced CORT secretion in male rats

These experiments aimed to assess the potential changes in basal CORT concentrations over 24 hours following acute SAFit2 or SAFit1 treatment. The hormone data obtained were analysed using the PULSAR algorithm (see section 2.15) to determine any changes in ultradian pulsatility. A further aim was to investigate the effects of SAFit2 and SAFit1 on stress-induced CORT secretion, which was done by exposing the rats to noise stress during the ABS.

For SAFit2 experiments rats were treated with either vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in 0.09% sterile saline; 2 ml/kg; s.c. n=8) or SAFit2 (20 mg/kg; s.c.; n=6) at 09.00h and 17.00h on the day of blood sampling. For SAFit1 experiments, rats were treated with either vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in 0.09% saline; 2 ml/kg; s.c. n=7) or SAFit1 (20 mg/kg; s.c.; n=6) at 09.00h, 14.00h and 19.00h on the day of blood sampling. Note that this experiment protocol differed from the SAFit2 experiment only in the times for the administration of the SAFit compounds, due to a shorter half-life of SAFit1 this compound was administered three times compared to two times for the SAFit2 (figure 3.1).

For both SAFit1 and SAFit2 experiments, automated-blood sampling (six samples per hour) for measurement of basal CORT was conducted from 07.00h on day 1, until 07.00h the following day. At 07.00h of day 2, all rats were subject to noise stress (10 min, 96dB, white noise) and blood sampling collection continued for one more hour. Immediately after the sampling had stopped at 08.00h, the rats were euthanized using pentobarbitone injection in the i.v. cannula, followed by decapitation with a guillotine. The brains were dissected to isolate specific areas, and, along with the anterior pituitary gland and adrenal glands, were snap-frozen on dry ice to be used for further gene expression analysis.

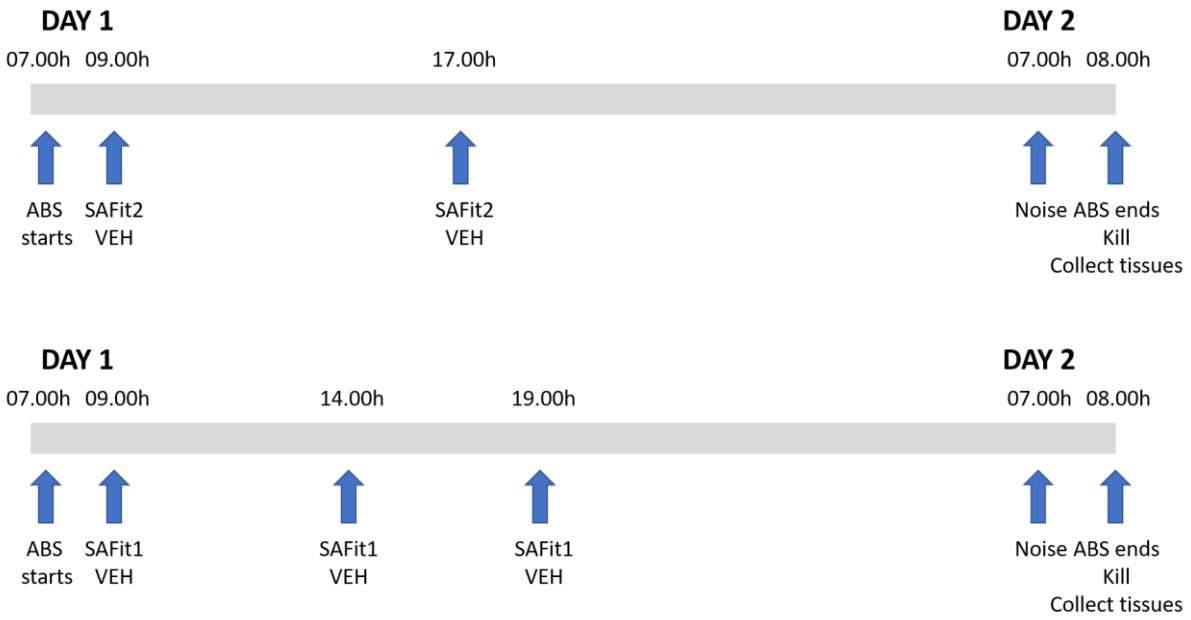

Figure 3.1. Experimental schedule for the acute SAFit2 and SAFit1 treatment in male rats.

3.2.2. Experiment 3: Effect of acute SAFit1 and SAFit2 on basal ACTH concentration in male rats

To determine whether the potential effect of SAFit2 and SAFit1 on CORT concentrations was reflected in the ACTH concentrations, this experiment aimed to investigate the effect of SAFit2 and SAFit1 on basal ACTH concentrations by manual sampling at regular intervals from 07.00h to 19.00h.

In this experiment, the rats were used as both the control group and the treated group. This was achieved by administering the vehicle to all rats on day 1 and administering either SAFit2 or SAFit1 to the rats on day 2. On day 1 of the experiment rats were treated with the vehicle at 09.00h and 14.00h (SAFit1 experiment, n=7), or 09.00h and 17.00h (SAFit2 experiment, n=7) and blood samples were taken manually from the jugular vein cannula at 09.00h, 11.00h, 13.00h, 15.00h, 17.00h and 19.00h. On day 2 of the experiment vehicle-treated rats were treated with SAFit1 at 09.00h and 14.00h, or with SAFit2 at 09.00h and 17.00h and blood samples were taken at the same time points as on day 1. From each sample, plasma was separated, and ACTH concentration was measured by RIA (see section 2.9).

3.2.3. Experiment 4: Effect of acute SAFit1 and SAFit2 on CRH-induced ACTH and CORT secretion in male rats

To investigate whether SAFit2 and SAFit1 exert any effects on ACTH the aim was to stimulate the anterior pituitary gland with CRH and measure the ACTH and CORT concentrations. This would allow me to demonstrate whether SAFit2 and SAFit1 have direct effects on the anterior pituitary gland.

This study was conducted using three groups, vehicle, SAFit1, and SAFit2. All three groups were acutely treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in 0.09% saline; 2 ml/kg; s.c.; n=5), SAFit1 (20 mg/kg/2 ml, n=4) or SAFit2 (20 mg/kg/2 ml, n=5) the day prior to the experiment. The SAFit1 was administered at 09.00h, 14.00h and 19.00h, and the SAFit2 was administered at 09.00h and 17.00h. To control for the difference in timing of the SAFit1 and SAFit2 treatments, vehicle was administered either at 09.00h, 14.00h and 19.00h or 09.00h and 17.00h; however, as further hormone measurements revealed that there was no effect of the timing of the vehicle treatment, the data were combined and used as one vehicle group. The CRH (150 ng/rat in 100 µl saline, i.v.) was injected via the cannula implanted in the jugular vein at 07.00h on the day of the experiment. Blood samples were collected manually from the implanted jugular vein cannula before CRH injection (0), and at 1, 5, 15, 30, and 60 minutes after the CRH injection. From each blood sample, plasma was separated and stored at -80°C. ACTH and CORT concentrations were measured by RIA (see section 2.8 and 2.9).

3.2.4. Experiment 5: Effect of acute SAFit1 and SAFit2 on ACTH-induced CORT secretion in male rats

To investigate whether SAFit2 and SAFit1 exert any effects on the adrenal glands, the aim was to stimulate the adrenal glands with ACTH and measure plasma CORT concentrations, to demonstrate whether the SAFit2 and SAFit1 have direct effects on the adrenal glands.

Three experimental groups were acutely treated with either vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in 0.09% saline; 2 ml/kg; s.c.; n=4), SAFit1 (2 mg/kg, s.c., at 09.00h, 14.00h and 19.00h, n=3), or SAFit2 (2 mg/kg, s.c., at 09.00h and 17.00h, n=5) the day prior to the experiment (day 1). At 07.00h on day 2, ACTH was injected in the intravenous cannula (40 ng per rat) and blood samples were collected manually from the implanted jugular vein cannula before ACTH injection (basal) and at 5, 15, 30, 45, and 60 minutes following ACTH injection. From each blood sample, plasma was separated and stored at -80. ACTH concentration was measured by RIA (see section 2.9).

3.2.5. Experiment 6: Effects of SAFit1 and SAFit2 on subcellular localisation and activation of GR and MR in the hippocampus and the anterior pituitary gland in male rats

Rats were injected subcutaneously with either SAFit1 (20 mg/kg/ml), SAFit2 (20 mg/kg/ml), or vehicle (4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg). After 60 minutes rats were injected with either vehicle (HBC in saline, 0.3 ml, i.p.) or CORT (0.3 mg/kg/ml HBC-CORT, i.p.) so that the experimental groups were the following: vehicle-HBC, vehicle-CORT-, SAFit1-CORT, and SAFit2-CORT. 30 minutes after CORT injection animals were sacrificed with isoflurane and their brains and pituitaries dissected. The hypothalamus, hippocampus, and anterior pituitary gland were processed using a kit for subcellular fractionations (Subcellular Protein Fractionation Kit, Thermo Fisher Scientific Inc., Rockford, IL, USA) to separate the tissue into the cytoplasmic, membrane, nuclear, and chromatin-bound extract (see section 2.13 in general methods). Western immunoblotting was used to quantify the GR and MR in each cellular extract from the different experimental groups. Phosphorylated GR (pGR) was quantified to investigate the effect of SAFit1 and SAFit2 on the activation of GR.

The 30 minutes post-CORT time point and the CORT dose used here (0.3mg/kg) is based on a previous study (359) which showed enhanced nuclear level GR in the hippocampus, hypothalamus and the anterior pituitary gland following the CORT injection. More importantly, 0.3mg/kg does not elicit a maximum translocation as demonstrated by further significant enhancement of nuclear GR following administration of 1mg/kg CORT (353). This is noteworthy as in this experiment the aim was to be able to see any increase in receptor translocation following SAFit2 and SAFit1 treatment and therefore it was important that the translocation in the vehicle -CORT group was not the maximum capacity.

3.3. Results

3.3.1. The effect of acute SAFit2 treatment on basal CORT concentrations and ultradian rhythms in adult male rats

Eight vehicle-treated rats and six SAFit2-treated rats were subjected to ABS every 10 minutes for 24 hours. The blood samples were assayed for CORT concentrations and the mean was calculated for each time point for both groups. The resultant average 24-h profiles are shown in figure 3.2 (A) along with the individual representative profile (B –E).

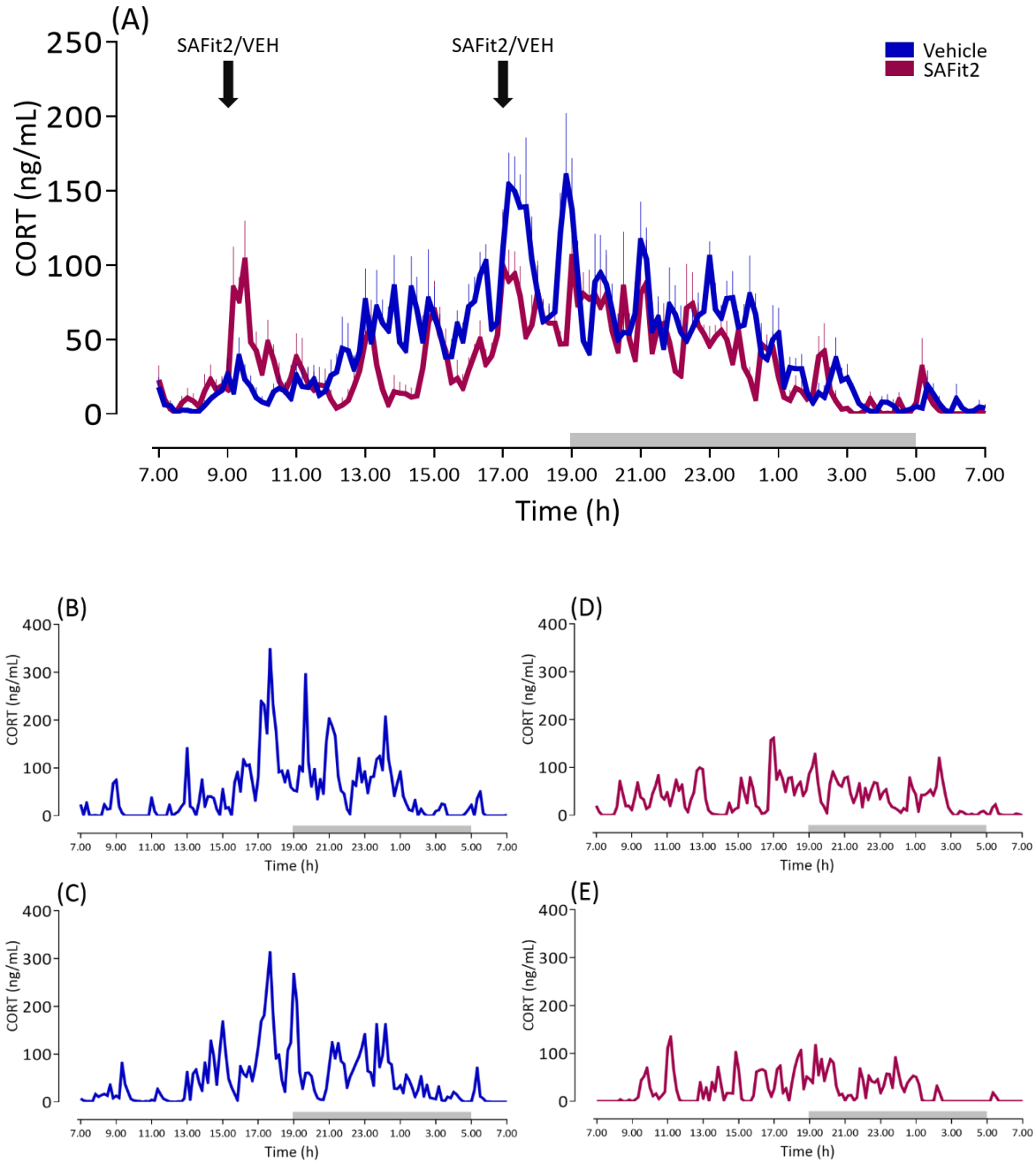

Figure 3.2. The effect of acute SAFit2 treatment on ultradian CORT secretion in male rats. *Mean + SEM (A) CORT concentrations, and representative individual profiles (B-E) from male adult rats treated with vehicle (B, C; VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=8) or SAFit2 (D, E; 20 mg/kg, s.c.; n=6) at 09.00h, and 17.00h. Blood samples were collected via a cannula implanted in the right jugular vein every 10 min for 24 hours using an automated blood sampling system. Plasma corticosterone was measured by radioimmunoassay (RIA). Grey bar indicates light off (19:00-05:00h).*

To allow for quantitative comparison of numerous parameters of CORT secretion, the PULSAR algorithm (see section 2.15) was used to analyse each corticosterone profile through the full 24-h period (figure 3.3). The PULSAR analysis and subsequent unpaired t-test revealed a significantly lower concentrations of maximum CORT in SAFit2-treated rats compared to vehicle-treated rats ($t(12)=2.21$, $P=0.047$). There was no significant effect of SAFit2 treatment compared to vehicle-treated rats on mean CORT ($t(12)=1.64$, $P=0.127$), baseline CORT ($t(12)=1.23$, $P=0.240$), number of pulses ($t(12)=-0.19$, $P=0.852$), pulse amplitude ($t(12)=1.52$, $P=0.154$), pulse height ($t(12)=1.52$, $P=0.154$), pulse length ($t(12)=0.29$, $P=0.777$), pulse area ($t(12)=1.49$, $P=0.161$), IPI ($t(12)=1.69$, $P=0.984$), pulse frequency ($t(12)=-0.19$, $P=0.852$), AUC basal ($t(12)=1.68$, $P=0.118$), or AUC total ($t(12)=1.65$, $P=0.125$).

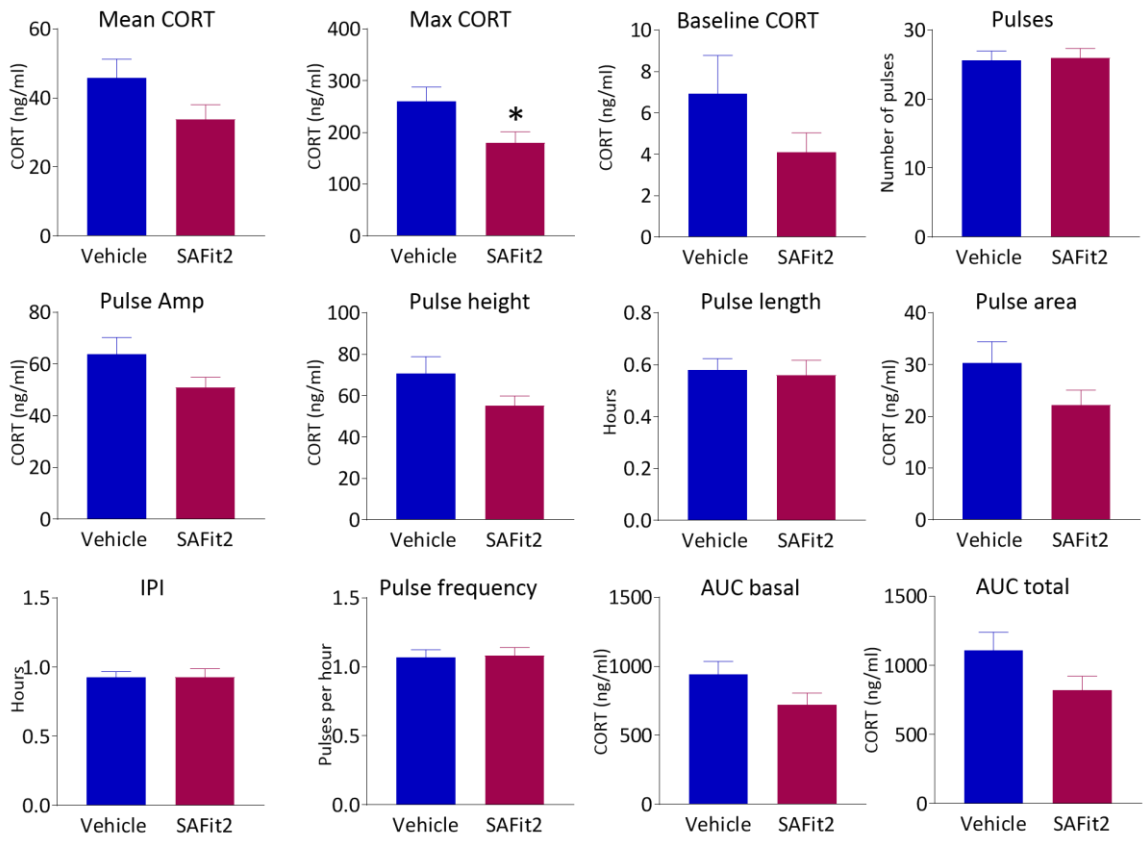

Figure 3.3. The effect of acute SAFit2 treatment on PULSAR parameters in male rats. *Mean + SEM CORT parameters calculated using PULSAR from 24-hour profiles of rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; n=8) or SAFit2 (20 mg/kg, s.c.; n=6) at 09.00h, and 17.00h. Amp=amplitude, IPI=inter-pulse interval, AUC= area under the curve. *P<0.05 compared to vehicle-treated rats, with unpaired t-test.*

These data indicate that inhibition of FKBP51 by SAFit2 in the rat decreases only the maximum CORT concentrations. This appears to be due to lowered pulse height and amplitude and hence reduce pulse area, although these were not significantly decreased in the SAFit2-treated rats compared to vehicle-treated rats. There was no effect on the inter-pulse interval or pulse length, indicating that SAFit2 does not alter the dynamic of each pulse of CORT.

Even though these data show that FKBP51 is involved in the regulation of CORT secretion in the rat, SAFit2 is active in the whole body, including the brain and the anterior pituitary gland, and therefore it cannot be deduced if inhibition of both central and peripheral

FKBP51 is required to reduce CORT concentrations. Furthermore, this study does not reveal at what level of the HPA axis the effects of SAFit2 occur.

3.3.2. The effect of acute SAFit1 treatment on basal CORT concentrations and ultradian rhythms in adult male rats

The same protocol as above was used to perform acute treatment with SAFit1, the peripheral-acting FKBP51 inhibitor, to further deduce the role of FKBP51 in regulating HPA axis activity, and to investigate if peripheral inhibition is sufficient to decrease basal CORT concentrations. The pituitary-adrenal gland pulse generator hypothesis (131, 132) described in section 1.4.4 of the introduction suggests that interactions between the adrenal gland and pituitary gland represent the main system responsible for maintaining the pulsatile pattern of CORT secretion. If we assume that SAFit1 is in very low concentrations in the brain, and is only active in the periphery, FKBP51 inhibition in the pituitary gland and/or the adrenal gland could alter the CORT pulsatility pattern.

Using the ABS, blood samples were collected every 10 min for 24h from 7 vehicle- and 6 SAFit1-treated male rats and the samples were assayed for CORT concentrations. Individual profiles from each experimental group are reported in figure 3.4 (B-E). The mean CORT concentration at each time point was calculated for both groups to produce a mean 24-h CORT profile, as shown in figure 3.4 (A).

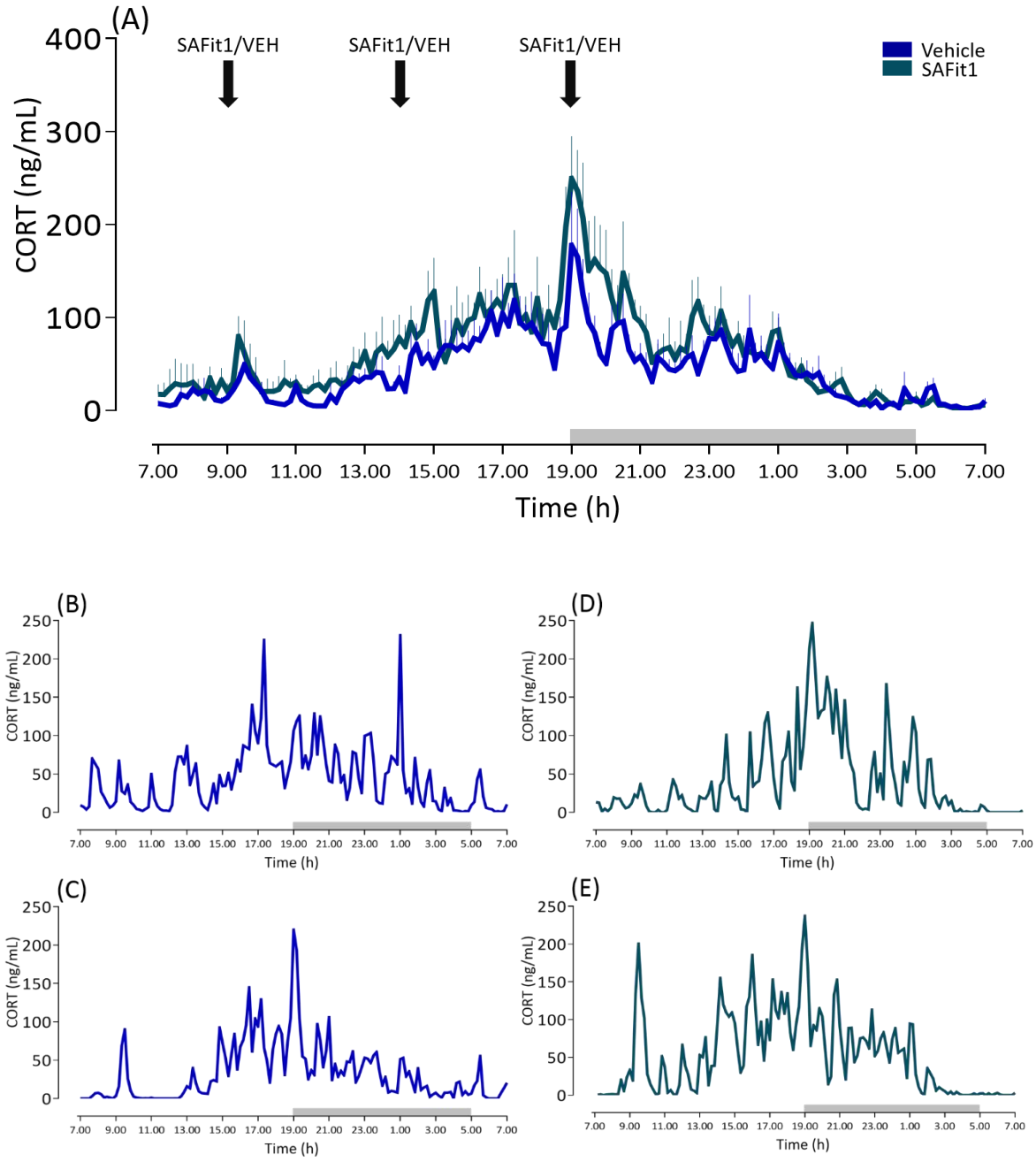

Figure 3.4. The effect of acute SAFit1 treatment on ultradian CORT secretion in male rats. *Mean + SEM (A) CORT concentrations, and representative individual profiles (B-E) from male adult rats treated with vehicle (B, C; VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=8) or SAFit1 (D, E; 20 mg/kg, s.c.; n=6) at 09.00h, and 17.00h. Blood samples were collected via a cannula implanted in the right jugular vein every 10 min for 24 hours using an automated blood sampling system. Plasma corticosterone was measured by radioimmunoassay (RIA). Grey bar indicates light off (19:00-05:00h).*

The PULSAR analysis and subsequent unpaired t-test of the 24-h CORT profiles (figure 3.5) revealed no effect of SAFit1 treatment compared to vehicle treatment on mean CORT ($t(11)=-1.35$, $P=0.202$), maximum CORT ($t(11)=-0.42$, $P=0.684$), baseline CORT ($t(11)=-0.77$, $P=0.452$), number of pulses ($t(11)=-0.03$, $P=0.974$), pulse amplitude ($t(11)=-1.45$, $P=0.173$), pulse height ($t(11)=-1.36$, $P=0.201$), pulse length ($t(11)=0.59$ $P=0.565$), pulse area ($t(11)=-0.51$, $P=0.616$), IPI ($t(11)=0.48$, $P=0.637$), pulse frequency ($t(11)=-0.03$, $P=0.974$), AUC basal ($t(11)=-1.37$, $P=0.197$), or AUC total ($t(11)=-1.25$, $P=0.236$).

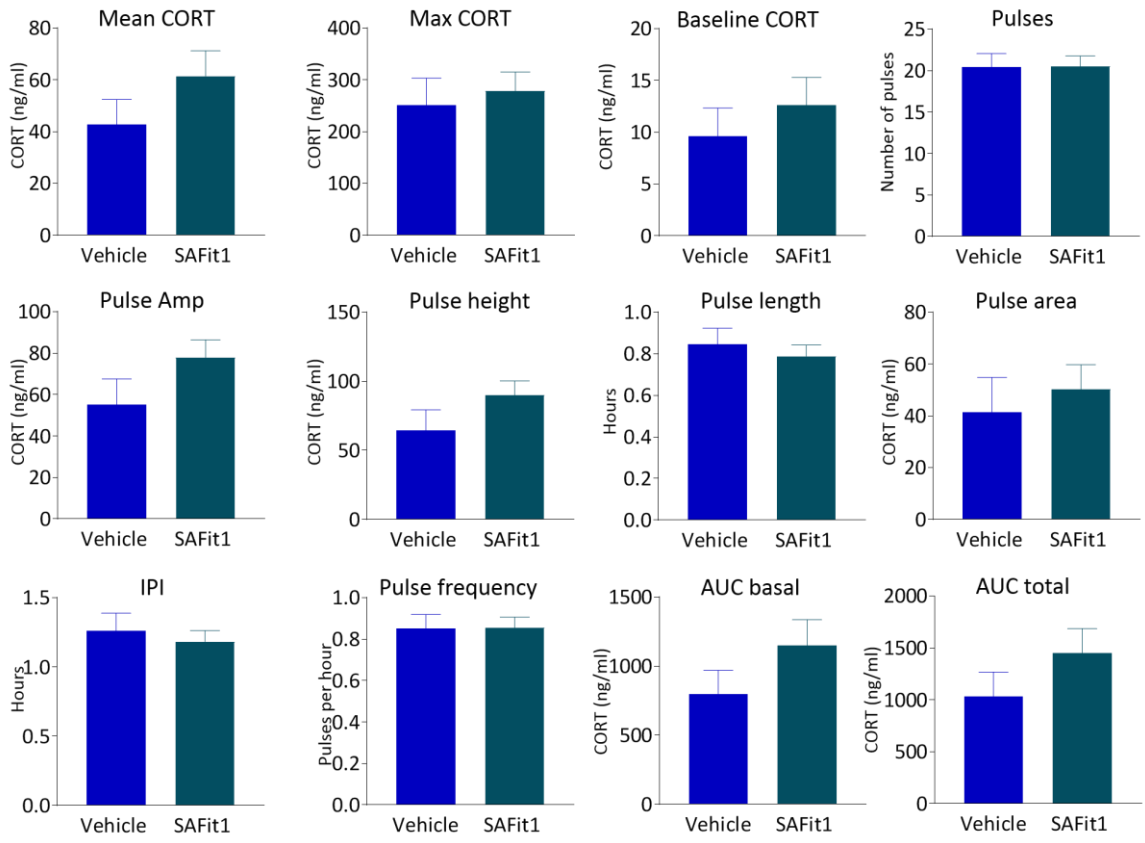

Figure 3.5. The effect of acute SAFit1 treatment on PULSAR parameters in male rats. *Mean + SEM CORT parameters calculated using PULSAR from 24-hour profiles of rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=7) or SAFit1 (20 mg/kg, s.c.; n=6) at 09.00h, and 17.00h. Amp=amplitude, IPI=inter-pulse interval, AUC= area under the curve.*

These data indicate that acute SAFit1 treatment does not affect CORT secretion under basal conditions, suggesting that peripheral-only inhibition of FKBP51 is not sufficient to generate the effects seen in the acute SAFit2 experiments. It is noteworthy that the shorter half-life of SAFit1 might contribute to the lack of effect, although the treatment protocol was adjusted to avoid complete clearance of SAFit1. Nevertheless, central inhibition of the FKBP51 is likely required for a reduction in basal CORT concentrations in male rats.

3.3.3. The effect of acute SAFit2 or SAFit1 treatment on stress-induced CORT secretion in adult male rats

Previous studies using the SAFit2 in male mice showed decreased CORT concentrations following a stressor whilst there were no effects on basal CORT (340). In the above study, significantly lower maximum CORT secretion was observed in the SAFit2-treated male rats, suggesting that there might be an effect on the stress response as well. Furthermore, another aim was to deduce whether SAFit1 would influence post-stress CORT secretion, considering that this compound is not centrally active. There is evidence for rapid pituitary gland negative feedback induced by GCs, however, it is generally accepted that the stress-induced negative feedback mechanism is mainly centrally regulated. For this experiment, the rats were all subject to a noise stressor, as this test has been used previously in our group and previous studies have shown it induces a robust but transient increase in CORT secretion in both male and female rats (239, 240). Furthermore, exposure to noise stress can be performed remotely (see general methods section 2.6.), hence without inducing further stress due to handling the rats.

Following 24-h blood sampling and treatment with SAFit2 or SAFit1, the rats were subjected to noise stress and blood sampling continued for 60 minutes following the noise stress (see schematic figure 3.1).

The mean CORT concentration at each time point was calculated for each group as shown in figure 3.6. In the SAFit2 experiment, repeated measure analysis showed an overall significant effect of time ($F(2.9, 34.2)=90.9$, $P<0.0001$), a significant interaction of time and treatment ($F(2.9, 34.2)=90.9$, $P=0.033$), and a significant effect of treatment ($F(1,12)=7.7$, $P=0.017$). Further, post hoc analysis revealed a significant increase in stress-induced CORT in

vehicle-treated rats at the 10 ($P<0.0001$), 20 ($P<0.0001$), 30 ($P<0.0001$), and 40 ($P<0.0001$) minutes time points, compared to vehicle-treated rats at the 0 minute time point. In comparison, a significant increase in stress-induced CORT in SAFit2-treated rats was observed at the 10 ($P<0.0001$), 20 ($P<0.0001$), and 30 ($P<0.0001$) minutes time points, compared to SAFit2-treated rats at the 0 minute time point. Moreover, there was a significant reduction in CORT secretion in the SAFit2-treated rats at the 20 minutes time point compared to vehicle-treated rats at the 20 minutes time point ($P=0.01$) (figure 3.6. A). There was no difference between the baseline CORT at the 0 minute time point between vehicle-treated rats and SAFit2-treated rats.

In the SAFit1 experiment repeated measures analysis showed an overall significant effect of time ($F(2.3, 23,1)=31.217$, $P<0.00001$), but no significant interaction between time and treatment ($F(2.3, 23,1)=1.638$, $P=0.214$), and no effects of treatment ($F(1,10)=0.009$, $P=0.928$). Further, post hoc analysis revealed a significant increase in stress-induced CORT in vehicle-treated rats at the 10 ($P<0.0001$), 20 ($P<0.0001$), and 30 ($P<0.0001$) minutes time points, compared to vehicle-treated rats at the 0 minute time point. Similarly, a significant increase in stress-induced CORT in SAFit2-treated rats was observed at the 10 ($P<0.0001$), 20 ($P<0.0001$), and 30 ($P<0.0001$) minutes time points, compared to SAFit1-treated rats at the 0 minute time point (figure 3.6. B). Further, there was no significant difference between the SAFit1-treated rats and the vehicle-treated rats at any of the time points. There was no difference between the baseline CORT at the 0 minute time point between vehicle-treated rats and SAFit1-treated rats.

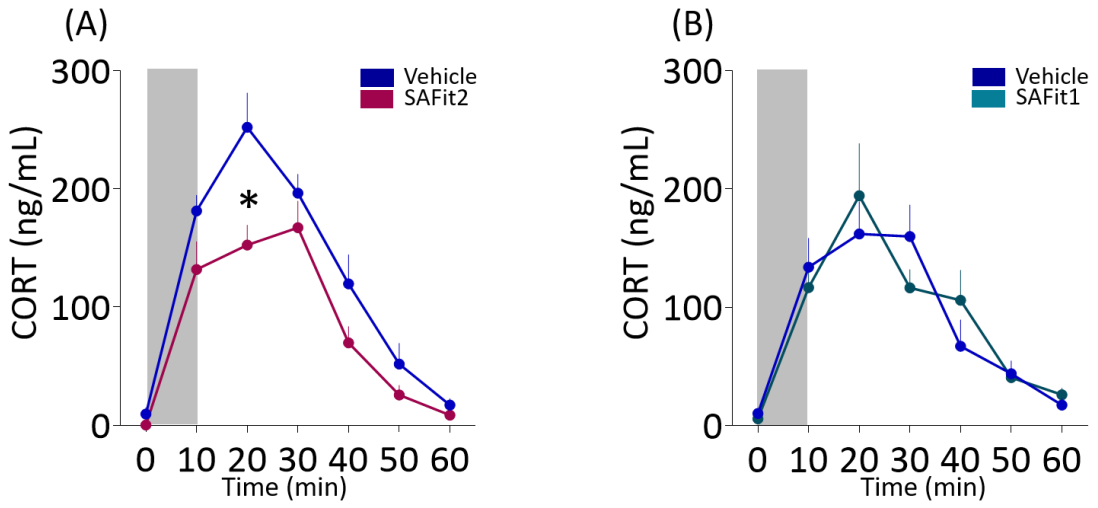

Figure 3.6. The effect of acute SAFit2 and SAFit1 treatment on stress-induced CORT secretion in male rats. *(A) Mean + SEM corticosterone levels during noise stress (96dB, 10 minutes, white noise, 07.00h) from male adult rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=8) or SAFit2 (20 mg/kg, s.c.; n=6) at 09.00h, and 17.00h on the day prior to the noise stress. (B) Mean + SEM corticosterone levels during a noise stress (96dB, 10 minutes, white noise, 07.00h) from male adult rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=7) or SAFit2 (20 mg/kg, s.c.; n=6) at 09.00h, 14.00h, and 19.00h on the day prior to the noise stress. Blood samples were taken every 10 minutes from a cannula implanted in the right jugular vein and CORT concentrations measured with CORT RIA. The grey bar indicates noise stress. *P<0.05; compared to rats treated with vehicle, with Tukey's post hoc test.*

These data indicate that there is an overall decrease in stress-induced CORT secretion in the SAFit2-treated group, while there was no difference between SAFit1-treated and vehicle-treated animals. This suggests that because the SAFit2 can cross the BBB it may enhance glucocorticoid-mediated negative feedback to reduce the stress response, in line with numerous studies suggesting a central regulation of the fast glucocorticoid negative feedback following a stressor. Even though the half-life of SAFit1 is shorter, any potential genomic effects of SAFit1 should still be present at the time of the noise stress, which was performed at 07.00h the day following treatment with SAFit1 or vehicle. It is also likely that treatment with SAFit2 may affect other areas of the brain involved in processing the stressor

effects, including for example the hippocampus and the amygdala (360) and that this might contribute to the different effects from the two SAFit compounds in these experiments. Studies have shown that, in addition to the PVN, several other areas are activated following audiogenic stress in rodents, these areas include the BNST, the lateral septum, some preoptic areas, and the orbitofrontal cortex (361). These areas all project to the PVN and hence may regulate HPA axis activity.

3.3.4. The effect of acute SAFit2 and SAFit1 treatment on basal ACTH concentrations in adult male rats

In the previous studies, it was shown that acute administration of SAFit2 decreases both basal and stress-induced CORT secretion in male rats. In contrast, there was no effect of acute SAFit1 treatment. This suggests a central regulation of the levels of CORT, as the main difference between the SAFit2 and SAFit1 is the penetration of the BBB, and SAFit1 is only active in the periphery. Enhanced central negative feedback by SAFit2 would reduce CRH concentrations and subsequently reduce ACTH secretion from the pituitary gland. To investigate this further, ACTH concentrations were measured over 12 hours, in animals treated with SAFit2 or SAFit1, or vehicle.

The CORT concentrations in the 24-h profiles were obtained from blood samples from the ABS. This is a remote system where the samples are stored at room temperature until the end of the sampling. However, because ACTH is not stable at room temperature for more than 6 hours (362), we cannot measure ACTH concentrations in these samples. Therefore, to measure ACTH concentrations, the animals must be sampled by hand, so the blood can instantly be separated into plasma and snap-frozen on dry ice. Moreover, the ACTH RIA used to measure the ACTH concentrations require 100ul of blood which is far greater than the volume sampled by ABS. Blood samples were taken from a cannula in the right jugular vein (see methods) every two hours from 09.00h to 19.00h. All rats were treated with vehicle and the following day with either SAFit2 or SAFit1. The samples were assayed for ACTH and CORT concentrations.

The mean + SEM ACTH concentrations for each time point was calculated for both groups as shown in figure 3.7. In the SAFit2 experiment (figure 3.7. A), repeated measures

revealed no effect of time (F(3.4,41.2)=1.2, P=0.333), no interaction of time and treatment (F(3.4,41.2)=0.1, P= 0.956), and no effect of treatment (F(1,12)=0.013, P=0.910). In the SAFit1 experiment (figure 3.7. B), repeated measures revealed a significant effect of time (F(3.8,45.5)=4.1, P=0.007), no interaction between time and treatment (F(3.8,45.5)=0.9, P=0.485), and no significant effect of treatment (F(1,12)=0.475, P=0.504). Tukey's post hoc test did not reveal any differences between the different time points.

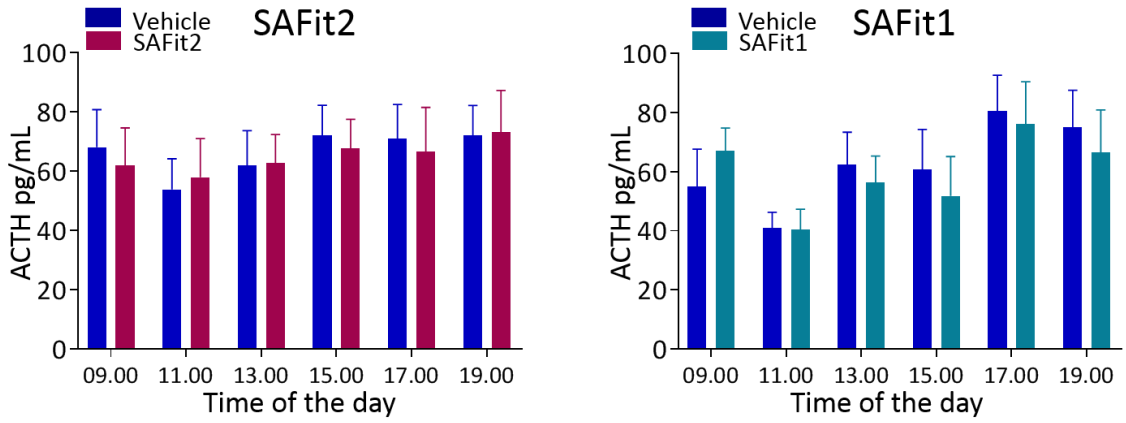

Figure 3.7. The effect of acute SAFit2 and SAFit1 treatment on basal ACTH concentrations in male rats. *Mean + SEM ACTH concentrations after treatment with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=4-6), SAFit1 (SF1; 20mg/kg; s.c.; n=5) or SAFit2 (SF2; 20mg/kg; s.c.; n=6). Blood was collected via a jugular vein cannula and plasma ACTH concentrations measured with ACTH RIA.*

Complete 24-hour CORT concentration profiles following acute SAFit2 and SAFit1 treatment in male rats have been established previously in this chapter. However, I wanted to see if the differences could be detected in these blood samples. The CORT concentrations were measured in all samples and are shown in figure 3.8. Repeated measures ANOVA for the SAFit2 experiment revealed an overall effect of time (F(2.9,35) =6.2,P=0.002), but not of treatment (F(2.9,35)=0.7,P=0.573), and there was no interaction (F(1,12)=0.020, P=0.889). Similar results were seen in the SAFit1 experiment, with an overall effect of time (F(2.5,24.6)=14.4,P=<0.00001), but not of treatment (F(2.5,24.6)=0.5,P=0.637), and no interaction (F(1,10)=0.035,P=0.856).

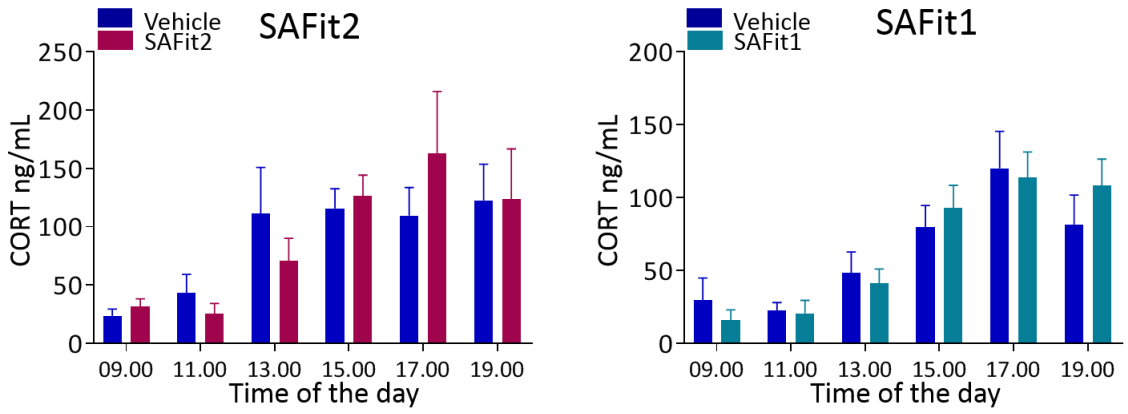

Figure 3.8. Comparison of CORT concentrations for basal ACTH measurements following acute SAFit2 and SAFit1 treatment in male rats. *Mean + SEM CORT concentrations after treatment with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=4-6), SAFit1 (SF1; 20mg/kg; s.c.; n=5) or SAFit2 (SF2; 20mg/kg; s.c.; n=6). Blood was collected via a jugular vein cannula and plasma CORT concentrations measured with CORT RIA.*

3.3.5. The effect of acute SAFit2 and SAFit1 treatment on CRH stimulation

In the previous experiment, no significant difference in the ACTH concentrations between the respective vehicle and SAFit1 or SAFit2-treated animals was detected. Therefore, to establish further whether the effects of SAFit2 on basal and stress-induced CORT secretion are mediated by decreased negative feedback at the level of the anterior pituitary gland corticotrophs, ACTH secretion was stimulated by injecting the rats with CRH. CRH binds CRHR1 in the anterior pituitary gland which causes the release of ACTH (12, 16), which in turn will increase the CORT production and release from the adrenal glands. To deduce which level of the HPA axis the SAFit1 and SAFit2 are affecting both the ACTH concentrations and the CORT concentrations were determined following an intravenous injection with 150 ng CRH per rat. Previous studies indicate a rapid increase in ACTH following CRH injection (363). If the SAFit1 and SAFit2 increase the negative feedback in the anterior pituitary gland, the same dose of CRH should result in a smaller ACTH increase in the SAFit-treated rats, when compared to vehicle-treated rats. If the SAFit1 and SAFit2 affect only the

negative feedback in the adrenal glands, we would expect no effect on ACTH concentration, however a decrease in CORT concentration.

In the stress experiments, a lower response to the noise stress was only observed in the SAFit2 group, suggesting that SAFit2 is acting centrally to reduce CRH. Further, noise stress is known to activate other brain areas in addition to the PVN (361). In the present experiment, the CRH was administered intravenously through a jugular vein cannula to minimise a stress response thus the central activation of endogenous CRH release. Moreover, the experiment was performed in the morning when CRH peptide stores are low (364) and therefore we would expect low basal endogenous levels of CRH and similar CRH levels following the CRH injection.

Rats were treated with SAFit2, SAFit1, or vehicle, and the following day all animals were injected with 150 ng CRH. Blood samples were manually taken from the rats before the injection (time 0 min) and then 1, 5, 15, 30, and 60 minutes following the CRH injection. The samples were assayed for ACTH and CORT concentrations. The vehicle was given at 09.00h and 17.00h, or at 09.00h, 14.00h, and 19.00h, to account for the different treatment times for SAFit2 and SAFit1. As there was no difference in hormone secretion within the vehicle groups data from all vehicle-treated rats were merged.

The mean ACTH concentrations following CRH administration are shown in figure 3.9. Repeated measures revealed a significant effect of time ($F(1.5,16.4)=4.1$, $P=0.046$), no interaction of time and treatment ($F(2.9, 16.4)=0.8$, $P=0.492$), and no effect of treatment ($F(1,13)=0.756$, $P=0.492$). Interestingly, the post hoc analysis revealed significantly higher ACTH concentrations in vehicle-treated rats at 15 ($P=0.005$), and 30 minutes time points ($P=0.009$), compared to vehicle-treated rats at the 0 minute time point. In comparison, there was no significant increase in ACTH at any of the time points for either SAFit2- or SAFit1-treated rats.

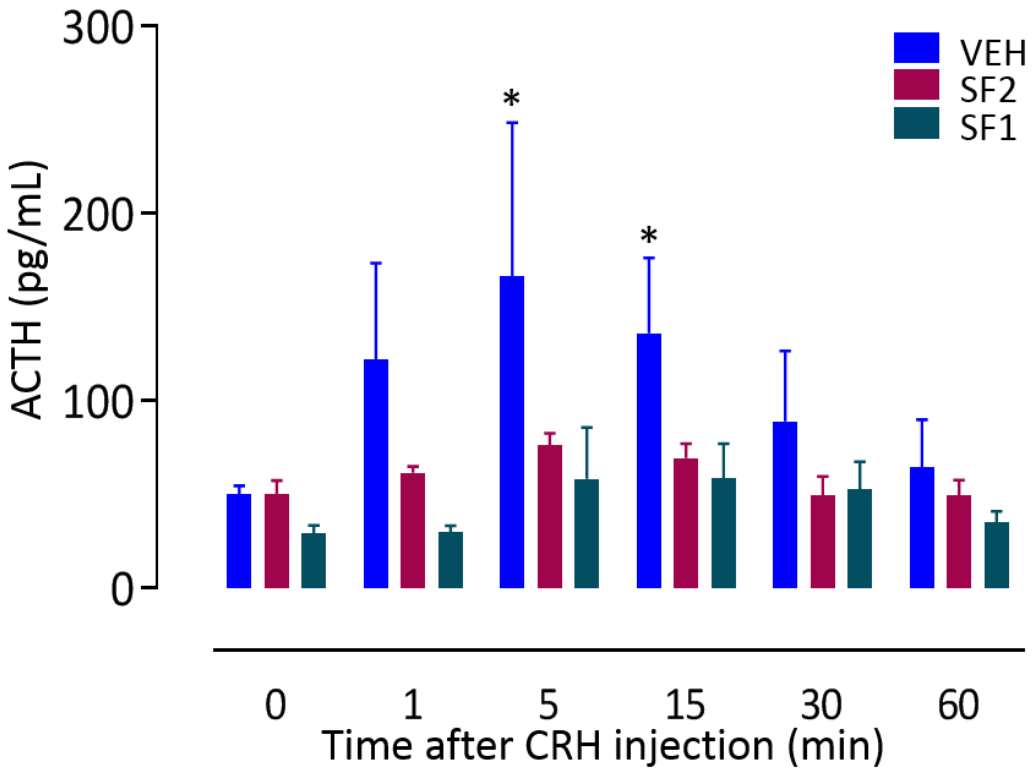

Figure 3.9. The effect of acute SAFit2 and SAFit1 treatment on CRH-induced ACTH secretion in male rats. *Mean + SEM ACTH concentration following CRH injection (150ng) in animals treated with Vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=6), SAFit2 (20 mg/kg, s.c.; n=6, at 09.00h and 17.00h), or SAFit1 (20 mg/kg, s.c.; n=4, at 09.00h, 14.00h, 19.00h) the day prior to CRH administration. Blood samples were collected via a jugular vein cannula and ACTH measured with ACTH RIA. *P<0.05 compared to respective 0 minute time point, with LSD.*

The mean CORT concentrations following CRH administration are shown in figure 3.10. Repeated measures revealed a significant effect of time ($F(2.6,33.6)=5.4$, $P=0.006$), but no interaction ($F(5.2,33.6)=0.8$, $P=0.535$), and no effect of treatment ($F(1,13)=2.292$, $P=0.140$). Interestingly, post hoc analysis revealed significantly higher CORT concentrations in vehicle-treated rats at the 15 ($P=0.012$), and 30 minutes ($P=0.012$) time points, compared to vehicle-treated rats at the 0 minute time point. In comparison, there was no significant increase in CORT at any of the time points for either SAFit2- or SAFit1-treated rats, compared to their

respective 0 minute time points. Further, significantly lower CORT concentration was observed in the SAFit1-treated rats at the 30 minute time point compared to vehicle-treated rats at the 30 minute time point. There was no difference between the baseline CORT at the 0 minute time point between vehicle-treated rats and SAFit2-treated rats, or between vehicle-treated rats and SAFit1-treated rats.

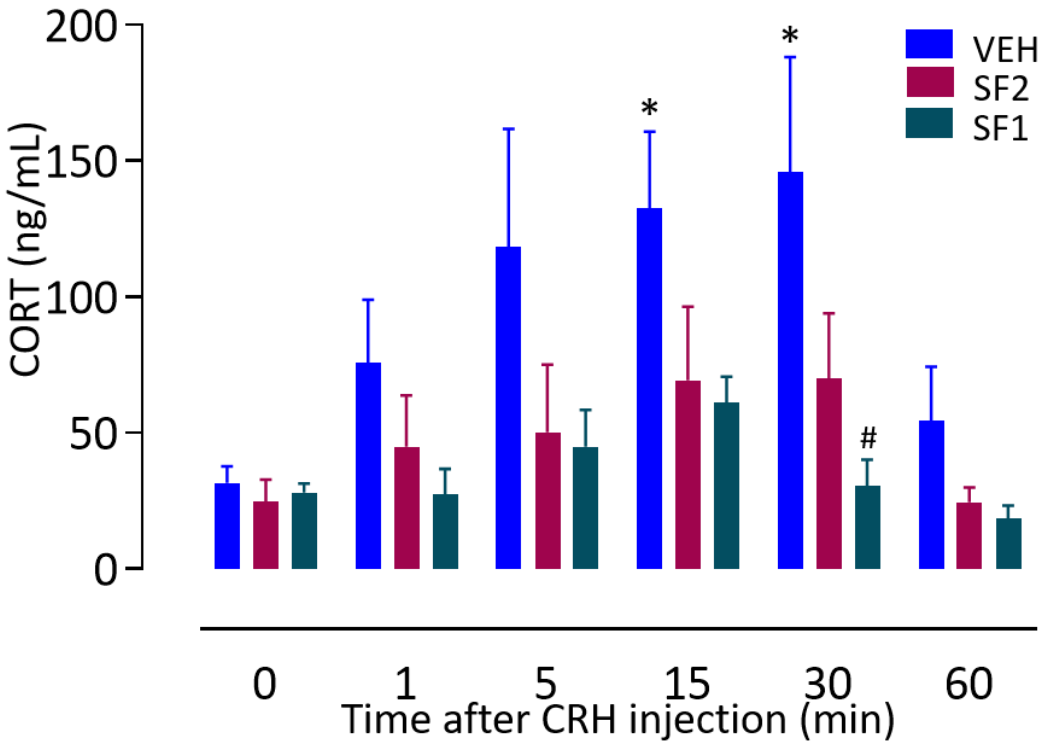

Figure 3.10. The effect of acute SAFit2 and SAFit1 treatment on CRH-induced CORT secretion in male rats. *Mean + SEM CORT concentration following CRH injection (150ng per rat) in animals treated with Vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=6), SAFit2 (20 mg/kg, s.c.; n=6, at 09.00h and 17.00h), or SAFit1 (20 mg/kg, s.c.; n=4, at 09.00h, 14.00h, 19.00h) the day prior to CRH administration. Blood samples were collected via a jugular vein cannula and CORT measured with CORT RIA. *P<0.05 compared to respective 0 minute time point, with LSD. #P<0.05 compared to vehicle-treated rats at the same time point, with LSD.*

The results from this experiment demonstrate a significant increase in both ACTH and CORT following CRH administration in the vehicle-treated rats only. This, in turn, indicates

that both SAFit2 and SAFit1 increase the GC negative feedback to significantly reduce the release of ACTH and subsequently CORT. Hence both SAFit2 and SAFit1 act in the anterior pituitary gland to reduce ACTH release following CRH stimulation. As GR is widely expressed in the anterior pituitary gland (365), it is likely that both SAFit2 and SAFit1, by inhibiting FKBP51, cause an increase in GR activity and thus increased GC negative feedback and lower ACTH concentrations.

3.3.6. The effect of acute SAFit2 and SAFit1 treatment on ACTH stimulation

In the previous section, the data demonstrated that acute administration of SAFit1 and SAFit2 before CRH injection did not cause an increase in ACTH concentrations, as seen in the vehicle-treated rats. This indicates that both SAFit1 and SAFit2 directly affect the secretion of ACTH, potentially by increasing the GR-mediated negative feedback at the level of the anterior pituitary gland corticotrophs. To investigate whether the lowered CRH-mediated CORT concentrations in SAFit1 and SAFit2 animals were due to lower ACTH concentrations, or whether SAFit1 and SAFit2 can affect the adrenal gland directly, the next experiment involved stimulation of the adrenal gland with ACTH and measuring the CORT concentrations.

The potential of GR to induce intra-adrenal gland negative feedback is not yet fully elucidated. Some evidence suggests that CORT-bound GR in the adrenal gland can bind the activity of the transcription factor SF-1. This causes induction of DAX-1 transcription, a negative regulator of StAR, the rate-limiting protein for CORT production in the adrenal gland (223). Furthermore, a recent study has shown, by using mathematical modelling, that activation GR can also affect the rapid synthesis, and thus secretion, of corticosterone in the adrenal gland (134). As FKBP51 is expressed in the adrenal gland, a potential inhibition of FKBP51 could affect GR activity and hence this proposed intra-adrenal gland rapid negative feedback.

The mean CORT concentrations following ACTH administration are shown in figure 3.11. Repeated measures revealed a significant effect of time ($F(2.1,18.9)=52.6$, $P<0.000001$), no effect of time and treatment interaction ($F(4.2, 18.9)=1.1$, $P=0.362$), and no effect of treatment ($F(1,9)=2.206$, $P=0.166$). Post hoc analysis revealed significantly higher CORT concentrations in vehicle-treated rats at 5 ($P<0.0001$), 15 ($P<0.0001$), and 30 minutes

(P=0.006) time points, compared to vehicle-treated rats at the 0 minute time point. Significantly higher CORT concentrations were observed in SAFit2-treated rats at 5 (P<0.0001), 15 (P<0.0001), and 30 minutes (P<0.0001) time points, compared to SAFit2-treated rats at the 0 minute time point. Further, significantly higher CORT concentrations were observed in SAFit1-treated rats at 5 (P=0.012), and 15 minutes (P<0.0001) time points, and a trend of effect at 30 minute (P=0.006) time point, compared to SAFit1-treated rats at the 0 minute time point. There was no difference between the baseline CORT at the 0 minute time point between vehicle-treated rats and SAFit2-treated rats, or between vehicle-treated rats and SAFit1-treated rats.

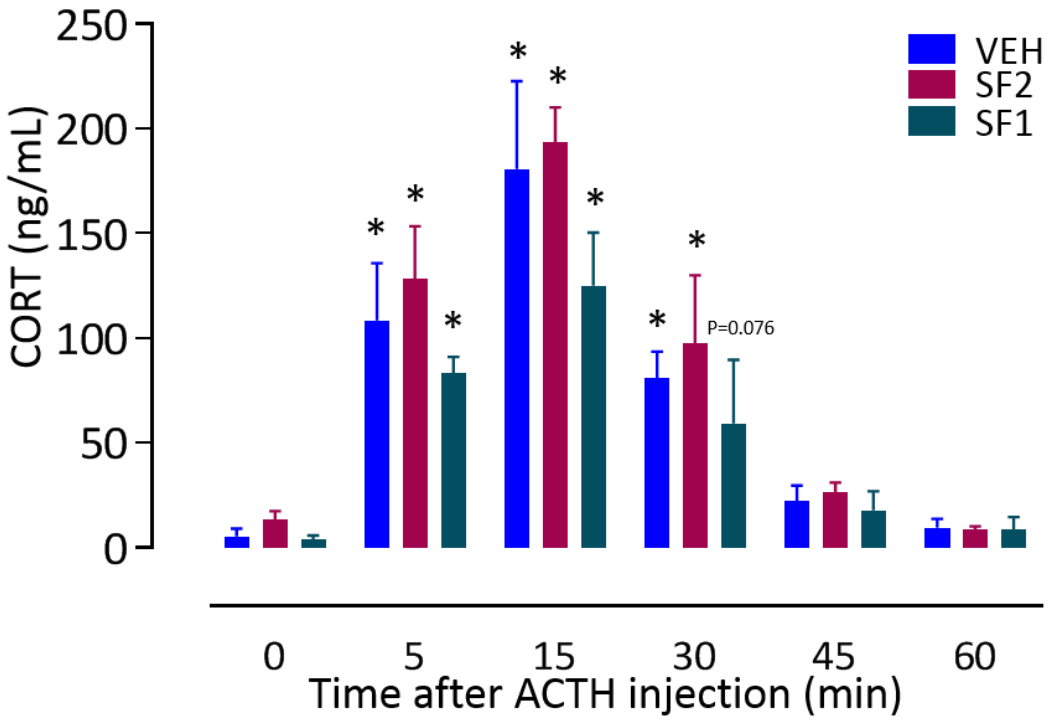

Figure 3.11. The effect of acute SAFit2 and SAFit1 treatment on ACTH-induced CORT secretion in male rats. *Mean + SEM CORT concentration following ACTH injection in animals treated with Vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=4), SAFit2 (20 mg/kg, s.c.; n=4, at 09.00h and 17.00h), or SAFit1 (20 mg/kg, s.c.; n=3, at 09.00h, 14.00h, 19.00h) the day prior to CRH administration. Blood samples were collected via a cannula implanted in the right jugular vein. *P<0.05 compared to respective 0 minute time point, with LSD.*

This is a very important finding in that it suggests that the site of action of the SAFit compounds responsible for the lower CRH-induced CORT secretion observed is in the anterior pituitary gland. It is not clear why SAFit2 and SAFit1, in this experiment, did not affect the ACTH-mediated CORT secretion from the adrenal glands. Even though it is hypothesised that an intra-adrenal gland GC negative feedback system exists, it is plausible that it is regulated differently than in the pituitary gland and the brain, hence why no effect of SAFit2 and SAFit1 were observed in this experiment. However, it should be noted that due to technical difficulties the animal numbers are low and further investigation will be needed to conclude whether SAFit2 and SAFit1 have any effects on GC-mediated negative feedback in the adrenal glands.

3.3.7. The effect of acute SASFit1 and SAFit2 treatment on subcellular localisation of GR and MR in the hippocampus and anterior pituitary gland of adult male rats

It is clear from the data reported in this chapter that inhibition of the FKBP51 has effects on the HPA axis activity, both under basal conditions and following stress. FKBP51 is a negative regulator of GR transcriptional activity by preventing the translocation of the receptor complex. Thus, inhibition of the FKBP51 by SAFit1 and SAFit2 is expected to enhance the nuclear levels of the GR. However, whether this is indeed occurring has not previously been tested. The FKBP51 is also a negative regulator of the MR, as well as of several protein kinases including GSK3B (328) and AKT (366) which both can regulate GR activity (367, 368). Therefore, this study aimed to further investigate the molecular effects of SAFit1 and SAFit2. Specifically, the effect of SAFit1 and SAFit2 on subcellular localisation of GR and MR following a CORT injection in adrenalectomized rats were investigated. This experiment was performed with the help of Miss Kajol Parikh.

In addition to nuclear localisation of the receptors, there is emerging evidence of cytoplasmic membrane-bound GR and MR (369) involved in the rapid effects of GC-mediated negative feedback both in the brain and the pituitary gland. The hypothesis suggests that SAFit1 and SAFit2 will enhance GR activity by inducing translocation of the receptor complex. However, as previously discussed, FKBP51 can also regulate kinases involved in mediating enhanced activity of the GR by phosphorylation of the receptor. In this study, Western

immunoblotting was used to measure GR, phosphorylated-GR, and MR levels in the membrane, cytoplasm, nucleus, and chromatin-bound protein extract.

To further characterise the differences between the SAFit1 and SAFit2 and their central *versus* peripheral effects GR and MR subcellular localisation in both the brain and the anterior pituitary gland were investigated. Unfortunately, due to technical difficulties in performing the subcellular fractionation, as well as the relatively low expression of GR and MR in the hypothalamus, no reliable data for GR and MR activation and subcellular localisation in the hypothalamus were produced. Instead, the hippocampus and anterior pituitary gland were used to distinguish between central and peripheral effects, respectively. Indeed, if there is an effect of SAFit1 or SAFit2 on CORT-induced sub-cellular translocation or activation of the GR and MR, these effects will be easier detected in a brain area with high GR and MR expression such as the hippocampus (66).

Firstly, to ensure that the levels of CORT were similar across the experimental groups at the time of kill, adrenalectomized animals were used. The adrenalectomy will remove endogenous CORT, which must be replaced by including CORT in the drinking water. Twelve hours before the experiment the CORT is removed from the drinking water to ensure the depletion of CORT. Corticosterone concentrations were measured in the trunk blood plasma, and the average values for each group are shown in figure 3.12. One-way ANOVA revealed a significant overall effect of the treatment ($F(3,18)=8.112$, $P=0.001$) with a robust increase of CORT in all three experimental groups, with similar CORT concentrations across the groups: 88.60 ng/ml in the vehicle-CORT, 65.97 ng/ml in the SAFit1-CORT, and 77.96 ng/ml in the SAFit2-CORT. The vehicle-SAL had average CORT concentrations of 23.40 ng/ml, which is higher than expected as these rats were adrenalectomized and should have no endogenous CORT. A similar study showed non-detectable CORT concentrations in the vehicle-saline group (359). The CORT concentration in the vehicle-saline group (figure 3.12) suggests that the adrenalectomy was only partial in some rats, and this should be taken into account when interpreting the results. The post hoc analysis did reveal significantly higher levels, compared to vehicle-SAL, in the vehicle-CORT group ($P=0.001$), in the SAFit1-CORT group ($P=0.029$), and in the SAFit2-CORT group (0.007), indicating that the CORT injection did cause an increase in CORT. Importantly, no significant difference in CORT levels was found between the three groups that were injected with HBC-CORT

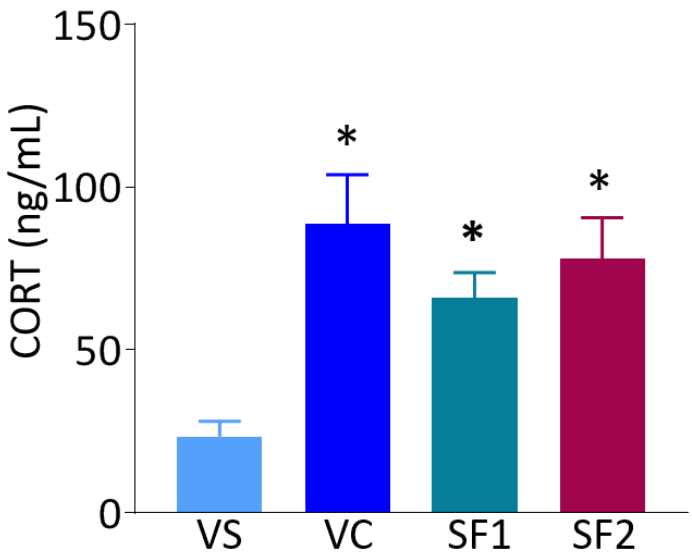

Figure 3.12. The effect of adrenalectomy on CORT concentrations. *Mean + SEM plasma CORT concentrations in Vehicle-Saline (VS, n=6), Vehicle-CORT (VC, n=5), SAFit1-CORT (SF1, n=6), and SAFit2-CORT (SF2, n=5) groups. Animals were treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.), SAFit1 (20mg/kg; s.c.), or SAFit2 (20mg/kg; s.c.), and 60 minutes later injected with saline (Vehicle-Saline; i.p.) or CORT (Vehicle-CORT, SAFit1-CORT, SAFit2-CORT; i.p.). *P<0.05 compared to Vehicle-Saline, with Tukey's post hoc test.*

The levels of GR, pGR, and MR were quantified in each sub-cellular extract for all animals using western immunoblotting. Figure 3.13 shows a representative blot of GR, MR, and pGR from each group in the hippocampus and the anterior pituitary gland sub-cellular extracts.

In the hippocampus there was no significant difference in GR or pGR levels between the groups in any of the sub-cellular extracts (figure 3.14). Further, two-way ANOVA revealed that the MR levels in the hippocampus cytoplasmic extract were significantly lower in the vehicle-CORT (P=0.004), in the SAFit1-CORT (P=0.009), and the SAFit2-CORT (P=0.013) groups, compared to vehicle-SAL. Although there was no significant effect on MR levels in any of the other extracts in the hippocampus, it would be expected that the MR leaves the cytoplasm and enters the nucleus. There is a large variability in the samples which is likely to account for

the unclear results. From the mean values it appears that there is an increase in the SAFit2-CORT group in nuclear MR, however, as this is not significant it is difficult to draw any conclusions. Work from Spiga and colleagues (359) has shown a robust GR translocation in the hippocampus 30 minutes following 0.3mg/kg CORT administration, but this is not seen in the present experiment. This might be due to the CORT concentrations in the CORT injected animals in our experiment were lower, and comparable to the 0.1mg/kg CORT group in the aforementioned study, where no significant effect on GR translocation in the hippocampus was seen.

In the anterior pituitary gland one-way ANOVA revealed a trend of effect on nuclear pGR levels (P=0.072), as shown by an increase in pGR in all three CORT groups. Moreover, there was also a trend of effect on chromatin-bound MR (P=0.093) (figure 3.15.). One-way ANOVA revealed no significant effects on GR, pGR, or MR in any of the other anterior pituitary gland sub-cellular extracts, however, the mean values suggest there is an increase in both nuclear GR and MR levels. Furthermore, although not significant, the mean values show an increase in membrane pGR in the anterior pituitary in the SAFit1 and SAFit2 groups (figure 3.15). However, it is difficult to draw any definite conclusions due to the large variability in the samples. A final observation, and perhaps the most interesting finding in this study, was the lack of any detectable MR in the membrane in the anterior pituitary. This is interesting because in the hippocampus MRs were present in the membrane, suggesting a differential cellular distribution of MR in the brain versus pituitary gland. It has been demonstrated that MR is present on the post-synaptic membrane in neurones in the amygdala (166), and synaptic GR in the hippocampus (154). It has also been shown that membrane GR and MR in the brain are involved in the regulation of synaptic structure and function, important in memory and learning (369). This study is consistent with previous reports of hippocampal GR and MR in the membrane.

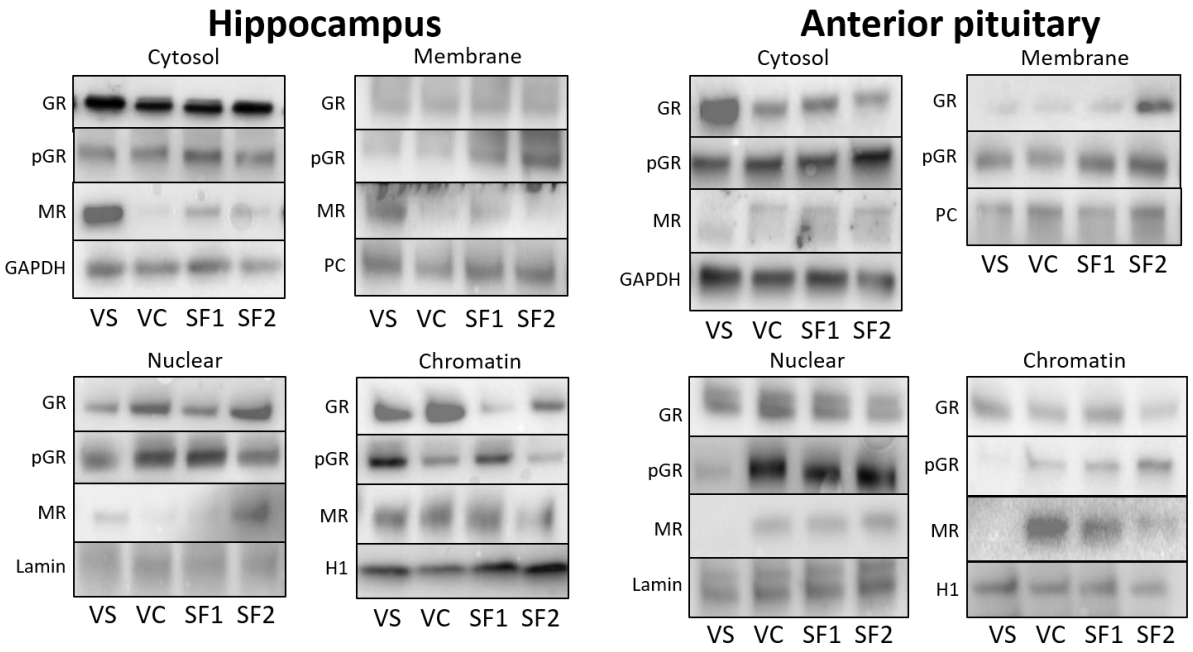

Figure 3.13. The effect of acute SAFit2 and SAFit1 treatment on GR and MR subcellular localisation. *Representative western immunoblotting images for the hippocampus, and anterior pituitary gland, for GR, pGR, and MR. Animals were treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.), SAFit1 (20mg/kg; s.c.), or SAFit2 (20mg/kg; s.c.), and 60 minutes later injected with saline-HBC (0.3 ml/kg, i.p.) or CORT-HBC (0.3 mg/kg; i.p.) to obtain the following groups: Vehicle-saline (VS, n=6), Vehicle-CORT (VC, n=5), SAFit1-CORT (SF1, n=6), and SAFit2-CORT (SF2, n=5). Size of the proteins: GR: ~90kDa, pGR: ~90kDa, MR: ~107kDa.*

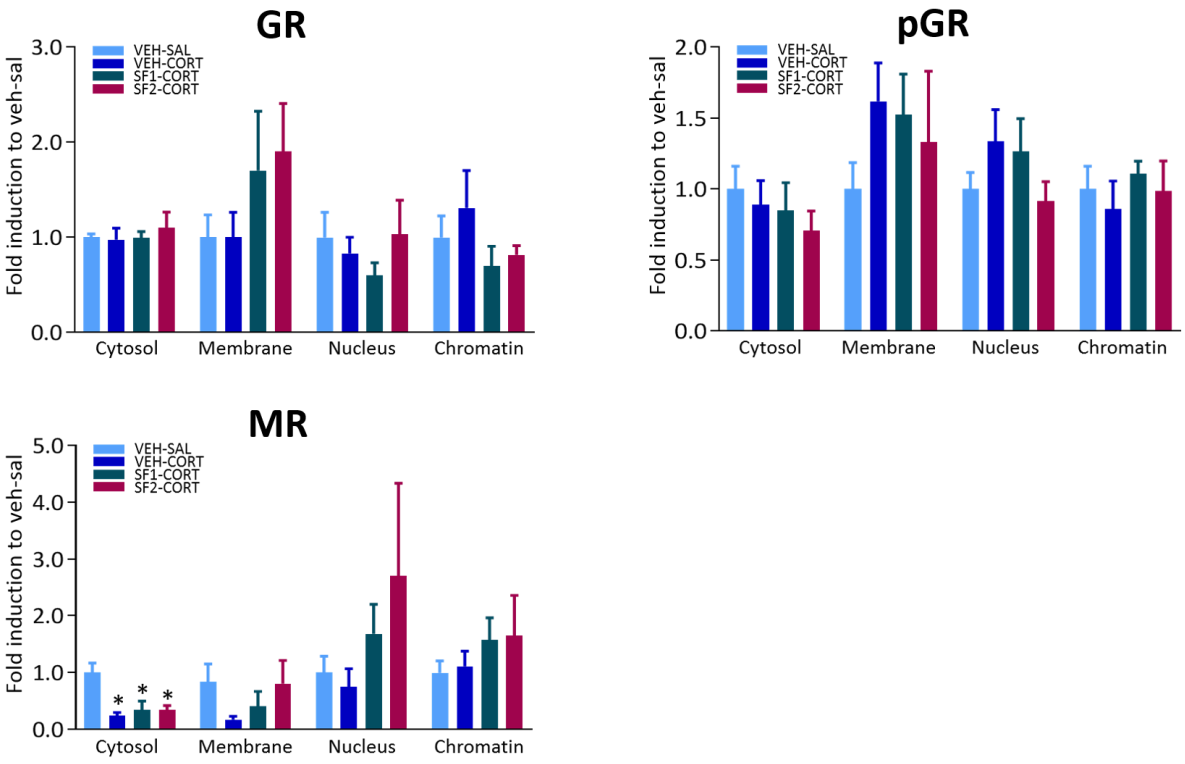

Figure 3.14. Quantification of the effect of acute SAFit2 and SAFit1 treatment on GR and MR subcellular localisation in the hippocampus. *Mean + SEM quantification of western immunoblotting images for hippocampal GR, pGR, and MR. Animals were treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.), SAFit1 (20mg/kg; s.c.), or SAFit2 (20mg/kg; s.c.), and 60 minutes later injected with saline-HBC (0.3 ml/kg; i.p.) or CORT-HBC (0.3 mg/kg; i.p.) to obtain the following groups: Vehicle-saline (VEH-SAL, n=6), Vehicle-CORT (VEH-CORT, n=5), SAFit1-CORT (SF1-CORT, n=6), SAFit2-CORT (SF2-CORT, n=5). *P<0.05 compared to vehicle-saline, with Tukey's post hoc test.*

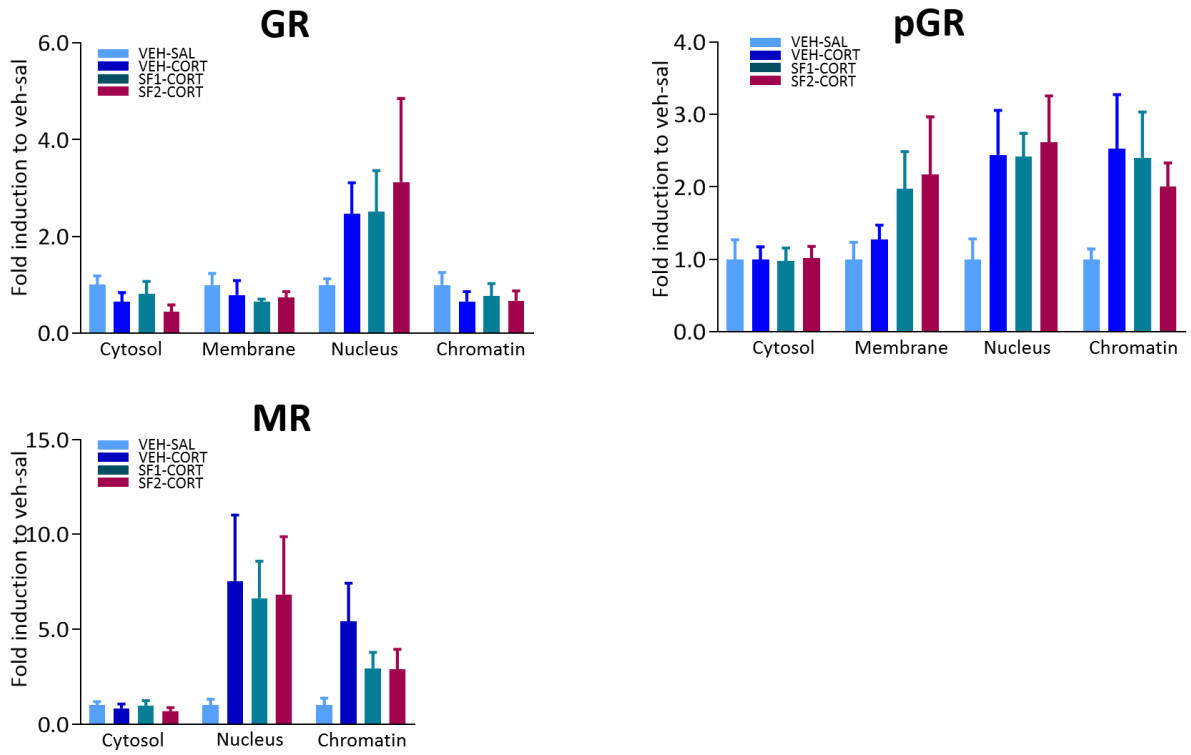

Figure 3.15. Quantification of the effect of acute SAFit2 and SAFit1 treatment on GR and MR subcellular localisation in the anterior pituitary gland. *Mean + SEM quantification of western immunoblotting images for GR, pGR, and MR in the anterior pituitary gland of animals treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.), SAFit1 (20mg/kg; s.c.), or SAFit2 (20mg/kg; s.c.), and 60 minutes later injected with saline-HBC (0.3 ml/kg; i.p.) or CORT-HBC (0.3 mg/kg; i.p.) to obtain the following groups: Vehicle-saline (VEH-SAL, n=6), Vehicle-CORT (VEH-CORT, n=5), SAFit1-CORT (SF1-CORT, n=6), SAFit2-CORT (SF2-CORT, n=5).*

3.3.8. Stress-induced gene expression in the hypothalamus and anterior pituitary gland following acute SAFit2 or SAFit1 treatment in male rats

To further investigate potential genomic changes induced by the SAFit2 and SAFit1 the gene expression was characterised following noise stress. Animals from the 24h sampling studies were subject to noise stress, and at 60 minutes post-noise they were euthanized, and their tissues dissected and processed for mRNA and subsequent RT-qPCR analysis. The expression of CRH and AVP was measured in the hypothalamus, and POMC in the anterior

pituitary gland, to investigate whether the SAFit2 or SAFit1 had any direct effects on these genes. It has been shown that in female rats, CRH mRNA in the hypothalamus is not increased at 4 hours post-noise (352). Another study showed that CRH mRNA only increased following chronic exposure to noise stress but not after acute exposure (370). To investigate whether there was a difference in AVP-induced ACTH secretion following SAFit administration, the AVP mRNA expression was measured. Furthermore, GR and MR mRNA in the hypothalamus and anterior pituitary gland was quantified to gain a better understanding of whether the SAFit compounds affect the expression of the receptors themselves. It has been shown that GR mRNA in the hypothalamus does not change following noise stress (370).

The GR is constitutively expressed in most tissues, and the levels vary as a result of, for example, neuroendocrine changes (371-373) and different stages of the cell cycle (374). The expression of GR is regulated by a variety of different pathways including GR autoregulation and second messengers such as cAMP. Glucocorticoids have been shown to downregulate the expression of GR (375) in human, rat, and mice cell lines, up to 8 hours following DEX administration, and that this downregulation is dependent on GR binding to GRE in its gene (376, 377). Since FKBP51 regulates GR transcriptional activity it is plausible that the SAFit compounds could affect GR expression.

Furthermore, the expression of c-Fos can be used as a marker for neuronal activity (378) and c-Fos expression is increased by exposure to stress and following noxious stimuli in rats (361). Therefore, c-Fos mRNA was measured in both the hypothalamus and the anterior pituitary gland following noise stress. Moreover, GILZ, PER1, SGK1, and FKBP5 itself are all well-known GR-inducible genes and hence can be used partly at least to establish GR transcriptional activity. The quantification of these genes was performed in the hypothalamus and the anterior pituitary gland as these areas are mainly involved in HPA axis activity. Other brain areas are involved in HPA axis negative feedback, for example, the hippocampus, however, due to time restraints, I chose to focus on the hypothalamus and the anterior pituitary gland.

The same experimental design was used for all ABS experiments, and hence the noise stress was performed at the same time, and rats sacrificed at the same time point, for all ABS experiments. The rats were sacrificed 60 to 90 minutes after the noise stress in order to measure the full CORT response during the stress, as previous studies indicate that the CORT

concentrations are back to basal concentrations at 60 minutes post-stress in both male and female rats (239, 379).

3.3.8.1. Hypothalamus

The relative expression of GR, MR, CRH, AVP, and c-Fos mRNA in the hypothalamus following the noise stress in the acute male SAFit2 experiment are shown in figure 3.15 A. An unpaired t-test revealed no effect of treatment on GR ($t(11)=0.05$, $P=0.954$), MR ($t(11)=-0.38$, $P=0.734$), CRH ($t(11)=-1.78$, $P=0.102$), AVP ($t(11)=1.07$, $P=0.306$), or c-Fos mRNA ($t(11)=-0.39$, $P=0.704$).

Furthermore, for the acute SAFit1-treated males, GR, MR, CRH, AVP, and c-Fos mRNA in the hypothalamus following noise stress are shown in figure 3.15 B. An unpaired t-test revealed an effect of treatment on MR ($t(10)=-2.417$, $P=0.036$), with higher relative expression in the SAFit1 group compared to the vehicle-treated group. There was no effect of treatment on GR ($t(10)=-0.454$, $P=0.659$), CRH ($t(10)=-1.06$, $P=0.314$), AVP ($t(10)=-0.13$, $P=0.896$), or c-Fos mRNA ($t(10)=0.37$, $P=0.707$), revealed by an unpaired t-test.

For the GR-inducible genes, FKBP5, GILZ, PER1, and SGK1 mRNA expression in the hypothalamus following the noise stress in the acute male SAFit2 experiment are shown in figure 3.15 C. There was no effect of treatment on FKBP5 ($t(11)=0.23$, $P=0.822$), GILZ ($t(11)=-0.41$, $P=0.689$), PER1 ($t(11)=0.22$, $P=0.826$), or for SGK1 mRNA ($t(11)=-0.24$, $P=0.812$) as shown by an unpaired t-test.

Figure 3.15 D shows the relative expression of FKBP5, GILZ, PER1, and SGK1 in the hypothalamus of acutely SAFit1-treated males following the noise stress. An unpaired t-test did not revealed any effect of treatment on either genes: FKBP5 ($t(10)=-0.93$, $P=0.373$), GILZ ($t(10)=-0.31$, $P=0.759$), PER1($t(10)=-0.43$, $P=0.674$), SGK1 ($t(10)=-0.43$, $P=0.676$).

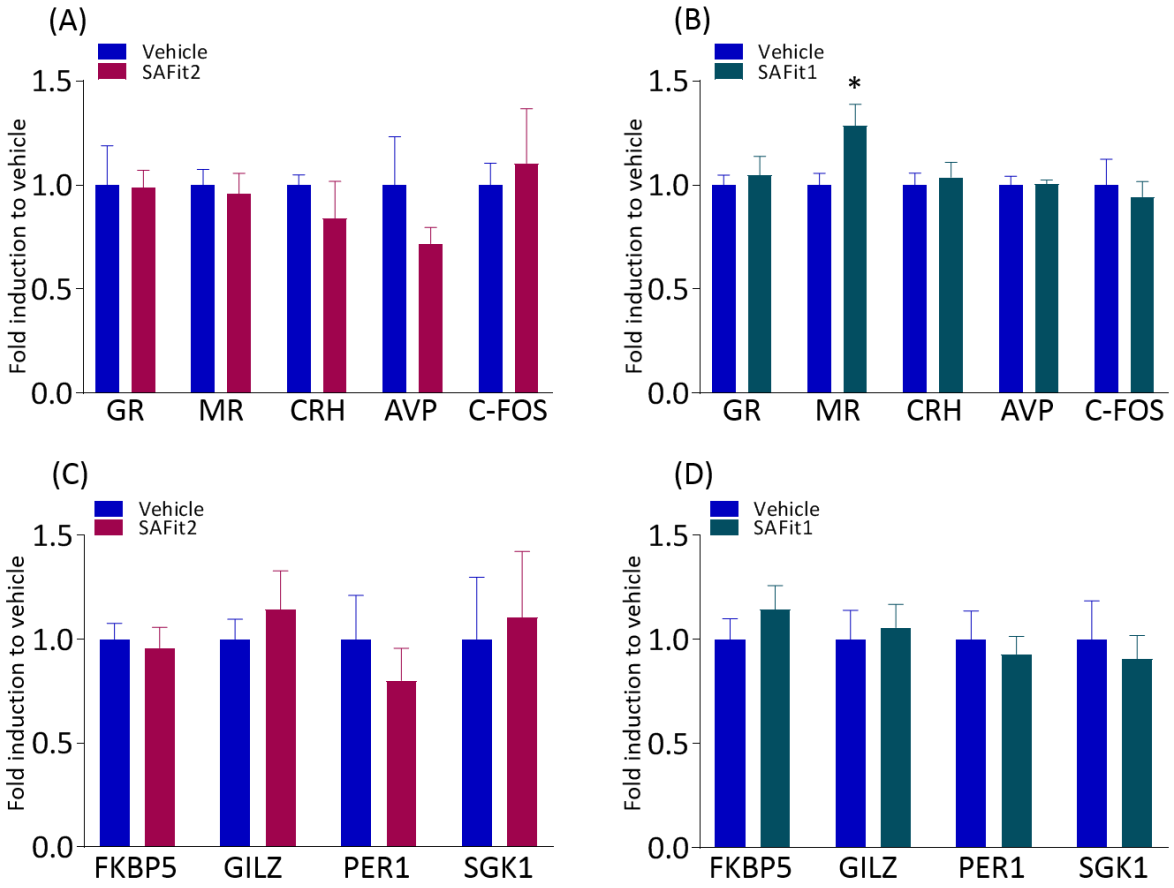

Figure 3.16. The effect of acute SAFit2 and SAFit1 on stress-induced gene expression in the hypothalamus of male rats. *Mean + SEM of GR, MR, CRH, AVP, and c-Fos mRNA in the hypothalamus of male rats treated with either vehicle or SAFit2 (A; n=6 and 8, respectively) or SAFit1 (B; n=7 and 6, respectively). Mean + SEM of FKBP5, GILZ, PER1, and SGK1 mRNA in the hypothalamus of male rats treated acutely with SAFit2 (C; n=6 and 8, respectively) or SAFit1 (D; n=7 and 6, respectively). *P<0.05 compared to vehicle-treated rats, with unpaired t-test.*

3.3.8.2. Anterior pituitary gland

The relative expressions of GR, MR, POMC, and c-Fos mRNA in the anterior pituitary gland following noise stress in the acute male SAFit2 experiment are shown in figure 3.16 A. An unpaired t-test did not show any effect of treatment on GR ($t(11)=-0.23$, $P=0.818$), MR ($t(11)=0.03$, $P=0.973$), POMC ($t(11)=-0.03$, $P=0.974$), or c-Fos mRNA ($t(11)=0.72$, $P=0.482$).

Similarly, in the acute male SAFit1 experiment, there was no effect of treatment on GR ($t(9)=-0.38$, $P=0.712$), MR ($t(9)=-0.75$, $P=0.468$), POMC ($t(9)=1.55$, $P=0.278$), or for c-Fos mRNA ($t(9)=0.107$, $P=0.917$), as revealed by an unpaired t-test (figure 3.16 B).

Quantification of the GR-inducible genes, FKBP5, GILZ, PER1, and SGK1 mRNA in the anterior pituitary gland following noise stress in the acute male SAFit2 experiment is shown in figure 3.16 C. Similar to the results from the hypothalamus, an unpaired t-test revealed no effect of treatment on FKBP5 ($t(11)=-0.15$, $P=0.883$), GILZ ($t(11)=-0.92$, $P=0.374$), PER1 ($t(11)=0.09$, $P=0.924$), or on SGK1 mRNA ($t(11)=-0.57$, $P=0.576$).

Further, relative expression of FKBP5, GILZ, PER1, and SGK1, in the anterior pituitary gland following noise stress in the acute male SAFit1 experiment is shown in figure 3.16 D. There was no difference in expression between the two groups, as shown by an unpaired t-test, for FKBP5 ($t(9)=-0.47$, $P=0.645$), GILZ ($t(9)=0.82$, $P=0.433$), PER1 ($t(9)=0.16$, $P=0.873$), or for SGK1 ($t(9)=0.93$, $P=0.375$).

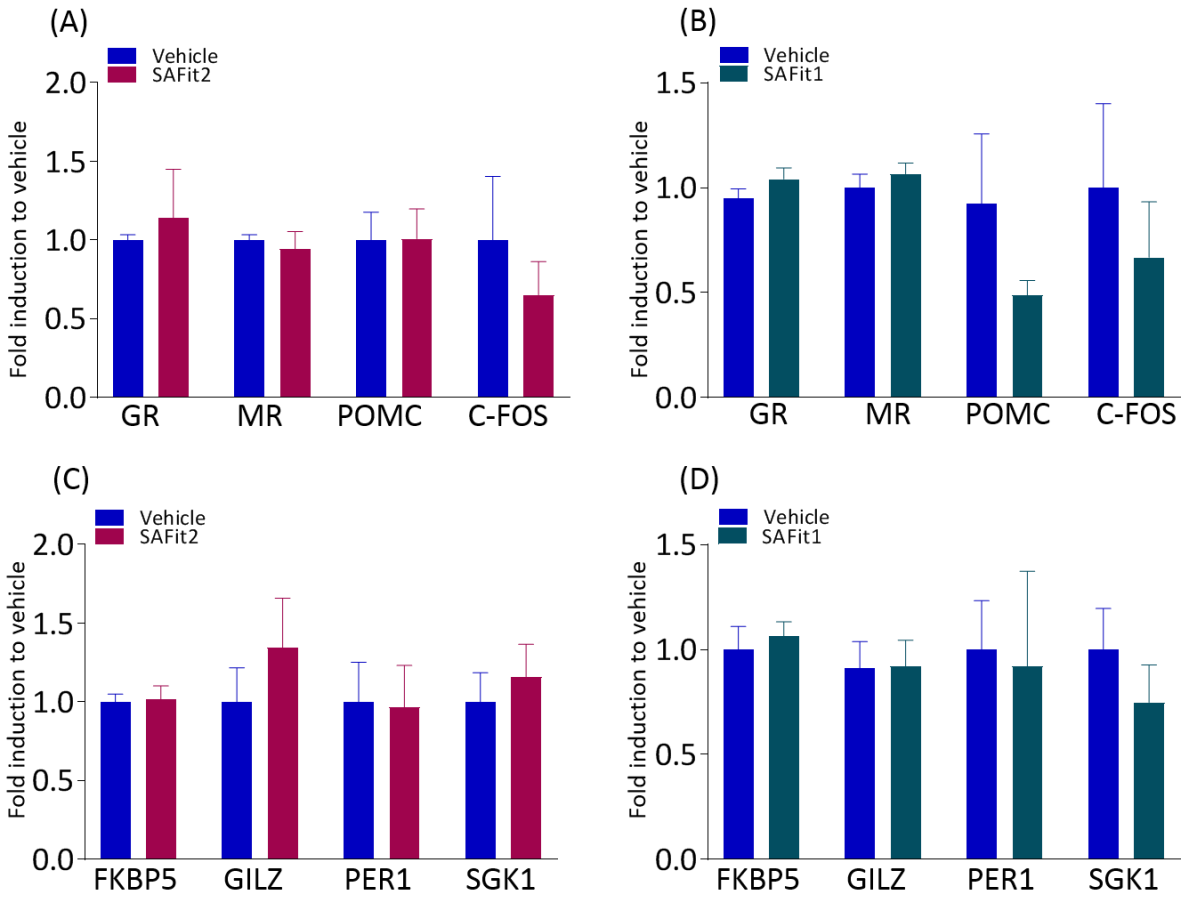

Figure 3.17. The effect of acute SAFit2 and SAFit1 on stress-induced gene expression in the anterior pituitary gland of male rats. *Mean + SEM of GR, MR, CRH, AVP, and c-Fos mRNA in the anterior pituitary of male rats treated with SAFit2 (A; n=6 and 8, respectively) or SAFit1 (B; n=7 and 6, respectively). Mean + SEM of FKBP5, GILZ, PER1, and SGK1 mRNA in the anterior pituitary gland of male rats treated acutely with SAFit2 (C; n=6 and 8, respectively) or SAFit1 (D; n=7 and 6, respectively). *P<0.05 compared to vehicle, with unpaired t-test.*

3.4. Discussion

In this chapter, data has been presented on the effect of acute SAFit2 and SAFit1 administration on basal and stress-induced HPA axis activity in adult male rats. It was demonstrated that acute SAFit2 administration decreased both max CORT and stress-induced CORT secretion, while there was no effect of acute SAFit1 treatment. This suggests that central inhibition of FKBP51 is required to affect CORT secretion in basal and stress-induced conditions, at least when the treatment is acute. It may also suggest that central and

peripheral FKBP51-inhibition has a stronger effect than peripheral-only FKBP51-inhibition, and perhaps a higher dose of SAFit1 would reduce CORT concentrations.

There was no difference in ACTH concentrations between vehicle and SAFit1 or SAFit2-treated groups at any of the time points investigated. This suggests that SAFit1 and SAFit2 have no major effect on ACTH production and/or secretion. However, it has been demonstrated in both rats and humans that ACTH is secreted in a pulsatile pattern (93) and therefore it is plausible that the rats were at different stages of the pulses when the blood samples were taken, which must be considered when analysing the present experiment. There may be a difference in ACTH concentrations between the groups, however not large enough to be detected with this experimental design. This experiment aimed to obtain an overall circadian profile of ACTH concentrations perhaps, however, more frequent blood sampling is necessary to detect a difference.

In the same experiment, there was an overall circadian difference in the CORT concentrations. In this experiment, however, there was no decrease in the SAFit2 group as seen in the ABS experiment. This is because the rats are not synchronised in their pulsatile pattern and therefore, they will be in different stages in their CORT ultradian pulses. Moreover, the decrease in the SAFit2 group in CORT concentrations in the ABS experiment was only significantly reduced in regards to the maximum CORT concentration, therefore it would be difficult to detect such decrease unless frequent sampling is performed. These data highlight the importance of performing complete 24-hour profiling of GC secretion to obtain potential differences subsequent a treatment that is aimed to alter HPA axis activity.

In the next experiment rats were acutely treated with SAFit2 or SAFit1 or vehicle and at 07.00h the following day (the same time as the noise stress in the ABS studies), all rats were injected with CRH (150ng per rat). This caused a stimulation of the pituitary gland corticotrophs and subsequent secretion of ACTH and CORT was induced. However, this increase in ACTH and CORT was only significant in the vehicle group, indicating that both SAFit2 and SAFit1 diminish the ACTH and subsequent CORT response to CRH. This suggests that the SAFit compounds induce a negative feedback mechanism to reduce the secretion of ACTH, and possibly CORT. As described above, there are various ways in which activated GR can affect the ACTH secretion, both via genomic pathways and non-genomic pathways. For example, activated GR may translocate to the nucleus, and in the anterior pituitary gland,

bind to the nGRE on the POMC gene to inhibit transcription of the ACTH precursor (214, 215). Moreover, non-genomic actions of CORT-activated GR includes regulation of the electropotential properties of the corticotropic cells in the anterior pituitary gland, and this can inhibit the vesicular secretion of ACTH (200). Secretion of ANXA1 from the nearby folliculostellate cells can act on the corticotrope cells and inhibit ACTH secretion by affecting the cytoskeleton and hence the vesicular transport of ACTH (208). Corticosterone may inhibit ACTH release by promoting the transport of ANXA1 from the cytoplasm to the plasma membrane (209). It is difficult to deduce whether the inhibitory effects of SAFit2 and SAFit1 on CRH-mediated ACTH secretion observed in this experiment are due to genomic or non-genomic GR actions. It is plausible that either of the pathways discussed above is activated by enhanced GR activity caused by FKBP51 inhibition following SAFit2 and SAFit1 treatment. There was large variability in the vehicle group and this variability in CRH response is expected as individual rats will secrete different amounts of ACTH and CORT in response to CRH stimulation. However, it is interesting to note that in the SAFit2 and SAFit1 groups, the variability is much smaller and all animals in the groups had lower ACTH and CORT concentrations compared to the vehicle groups. This suggests that indeed SAFit2 and SAFit1 are effective in lowering CRH-mediated ACTH release, and in turn lower ACTH-mediated CORT release.

In contrast, there was no difference in the CORT response from ACTH-mediated stimulation of the adrenal gland. This suggests that neither SAFit2 nor SAFit1 affects the adrenal gland directly to reduce CORT secretion. Hence, the lower CORT concentrations in the CRH experiment are expected to be due to lower ACTH secretion.

The results from the CRH- and ACTH-injection experiments indeed suggest an anterior pituitary gland effect of both SAFit2 and SAFit1. Future experiments to confirm these results could include using pituitary gland primary cell cultures from rats or using the pituitary corticotrophs cell line, AtT-20. These cell cultures can be pre-treated with SAFit2, SAFit1, or vehicle. Once CRH is added the response can be measured with, for example, calcium imaging, or by measuring the ACTH concentrations directly. In addition, a time course of pre-treatment with the SAFit2 or SAFit1 can reveal whether the effects are genomic or non-genomic. For example, if the cells are pre-treated with SAFit2 or SAFit1 5 minutes before CRH is added, and

the response in ACTH is diminished compared to the vehicle-treated cells, these rapid actions could indicate a non-genomic effect.

Finally, to elucidate the molecular mechanisms behind these results, the subcellular localisation of GR and MR in the hippocampus and anterior pituitary gland was quantified, with no clear results.

The data reported in this chapter demonstrate that global FKBP51-inhibition by SAFit2 is effective in reducing both maximum CORT concentrations and stress-induced CORT secretion. This effect appears to be due to lower ACTH secretion following CRH stimulation of the corticotrophs in the anterior pituitary gland.

There were some limitations to the studies in this chapter. For the CRH and ACTH injection experiments, the animal numbers in each group were low, due to difficulty maintaining the jugular vein cannula patency throughout the experiment and limited availability of the SAFit2 and SAFit1 compounds.

Furthermore, in the molecular mechanism experiment, the adrenalectomy appeared to be only partial in some of the rats, which could have contributed to variable results as it will affect the endogenous CORT concentrations. In addition, the technique used to quantify the nuclear translocation and the subcellular localisation in the same experiment was perhaps not sensitive enough to detect differences. Possibly a more suitable option would have been to perform chromatin immunoprecipitation (ChIP) on hypothalamic and anterior pituitary gland tissue from rats treated with either SAFit2, SAFit1, or vehicle and injected with CORT to stimulate GR translocation. Chromatin immunoprecipitation is used to investigate protein-DNA interactions *in vivo* and can detect for example direct protein-DNA binding, histone modifications, and cofactors associated with the DNA (380). This could be a useful technique to determine whether SAFit2 and SAFit1 affect GR interactions with for example DNA or cofactors.

Other limitations include a single time point for the gene expression analysis following the noise stress. The animals were euthanised at 60 minutes post-noise and the tissues were extracted and processed for RT-qPCR. It would have been ideal to have multiple time points, for example, 60, 120, and 180 minutes post-noise to obtain a better profile of the gene expression. Another study using foot-shock as a stressor found that both PER1 and SGK1

mRNA were significantly increased at 60 minutes post-stress in the ventral hippocampus, however, FKBP5 mRNA was only significantly increased at 180 minutes post-stress (355). The same study showed that hnRNA of all three genes showed a more robust increase than mRNA, and peaked at 30 minutes for PER1 and SGK1 and 60 minutes for FKBP5, in the ventral hippocampus (355). The CORT concentrations peaked over 1000 ng/ml at 30 minutes post-foot-shock in this experiment, compared to between 300-400 ng/ml in my noise stress experiment. It is plausible that an increase in the GR inducible genes would have been observed following higher CORT concentrations. Another study using female rats in pro-oestrus and oestrus showed a robust increase in CRH mRNA at 60 minutes following restraint stress (13), although the CORT concentrations were around 1500 ng/ml at 60 minutes post-stress which is substantially higher than in my experiments following noise stress. In summary, previous studies have shown a robust increase post-stress in some of the genes investigated in the present study (13, 355).

Although more time points are needed to investigate the time course of stress-induced expression of CRH, POMC, AVP, PER1, SGK1, GILZ, and FKBP5 in the hypothalamus and anterior pituitary gland that was beyond the scope of this thesis. It was simply not possible because a limited amount of SAFit2 and SAFit1 were available and to have included one more time point would have required twice as many animals. Nevertheless, a more suitable technique for analysing the gene expression, which would also produce an anatomical representation of the genes, is in situ hybridization (ISH). This technique uses labelled complementary DNA or RNA probes to identify the presence of the gene of interest (381) on, for example, brain slices. Moreover, recent advancement of ISH, RNA scope allows for signal amplification and simultaneous background noise suppression to allow for single-molecule detection (382).

Chapter 4: Prolonged treatment with FKBP51 inhibitors in male rats

4.1. Introduction

Acute administration of the central and peripheral FKBP51-inhibitor, SAFit2, results in decreased basal and stress-induced CORT concentrations in male rats. However, it is not known whether these effects are enhanced after prolonged treatment with SAFit2. Furthermore, as the peripheral-only FKBP51 antagonist SAFit1 did not have any effect on either the basal or stress-induced CORT concentrations, it is plausible that a sub-chronic treatment with SAFit1 necessary to see an effect on HPA axis activity.

In this chapter data on the effect of sub-chronic treatment with SAFit2 and SAFit1 on basal CORT, ultradian pulsatility, and stress-induced CORT secretion will be shown. Furthermore, the effects of prolonged treatment with SAFit2 and SAFit1 on gene expression related to the HPA axis and GR-induced gene expression will be demonstrated.

Previous studies using a GR antagonist show enhanced effects in increasing CORT concentrations when the antagonist was delivered sub-chronically compared to acute administration (383). This suggests that prolonged treatment with a GR modulator will have stronger effects than when delivered acutely.

4.2. Methods

4.2.1. Experiments 7 and 8: Effect of sub-chronic SAFit2 and SAFit1 treatment on CORT ultradian rhythm and stress-induced CORT secretion in male rats.

These experiments aimed to investigate whether prolonged treatment with SAFit2 and SAFit1 had different effects on HPA axis activity than acute treatment.

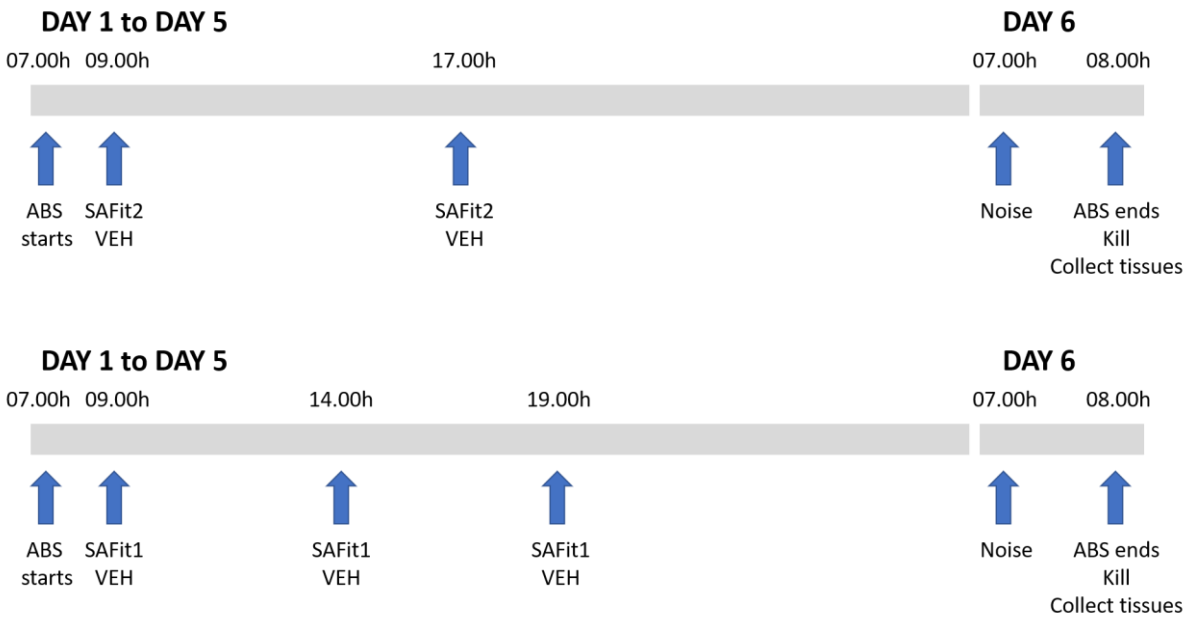

Figure 4.1. Experimental schedule for the sub-chronic SAFit2 and SAFit1 treatment in male rats.

For SAFit2 experiments rats were treated with either vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in 0.09% sterile saline; 2 ml/kg; s.c. n=7) or SAFit2 (20 mg/kg; s.c.; n=6) at 09.00h and 17.00h for 5 consecutive days, with the fifth day on the day of blood sampling. For SAFit1 experiments, rats were treated with either vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in 0.09% saline; 2 ml/kg; s.c. n=8) or SAFit1 (20 mg/kg; s.c.; n=6) at 09.00h, 14.00h and 19.00h 5 consecutive days, with the fifth day on the day of blood sampling. Note that this experiment protocol differed from the SAFit2 experiment only in the times for the administration of the SAFit compounds, due to a shorter half-life of the SAFit1 this compound was administered three times compared to two times for the SAFit2.

For both SAFit1 and SAFit2 experiments, automated-blood sampling for measurement of basal CORT was conducted from 07.00h on day 5, until 07.00h the following day. At 07.00h of day 6, all rats were subject to noise stress (10 min, 96dB, white noise) and blood sampling collection continued for one more hour. Immediately after the sampling had stopped at 08.00h, the rats were euthanized using pentobarbitone injection in the i.v. cannula, followed by decapitation with a guillotine. The brains were dissected to isolate specific areas, and,

along with the anterior pituitary gland and adrenal gland, were snap-frozen on dry ice to be used for further gene expression analysis.

4.3. Results

4.3.1. The effect of sub-chronic SAFit2 treatment on basal CORT concentrations and ultradian rhythms in adult male rats

Seven vehicle-treated rats and six SAFit2-treated rats were treated for 5 consecutive days and on the fifth day they were all subject to ABS every 10 minutes for 24 hours. The blood samples were assayed for CORT concentrations and the mean was calculated for each time point for both groups. The resultant average 24-h profiles are shown in figure 4.2 (A) along with the individual representative profile (B-E).

All the individual 24-hour profiles were analysed with PULSAR and the means for each parameter are shown in figure 4.3. Unpaired t-tests revealed a significant decrease in mean CORT ($t(11)=2.86$, $P=0.014$), max CORT ($t(11)=3.44$, $P=0.006$), pulse amplitude ($t(11)=2.47$, $P=0.031$), pulse height ($t(11)=2.51$, $P=0.029$), pulse area ($t(11)=2.30$, $P=0.042$), AUC basal ($t(11)=2.73$, $P=0.017$), AUC total ($t(11)=2.72$, $P=0.020$) in the SAFit2-treated rats compared to vehicle-treated rats. There was no significant effect on basal CORT ($t(11)=1.57$, $P=0.143$), the number of pulses ($t(11)=0.344$, $P=0.738$), the pulse length ($t(11)=0.837$, $P=0.421$), the IPI ($t(11)=-0.29$, $P=0.770$), or the pulse frequency ($t(11)=0.344$, $P=0.780$).

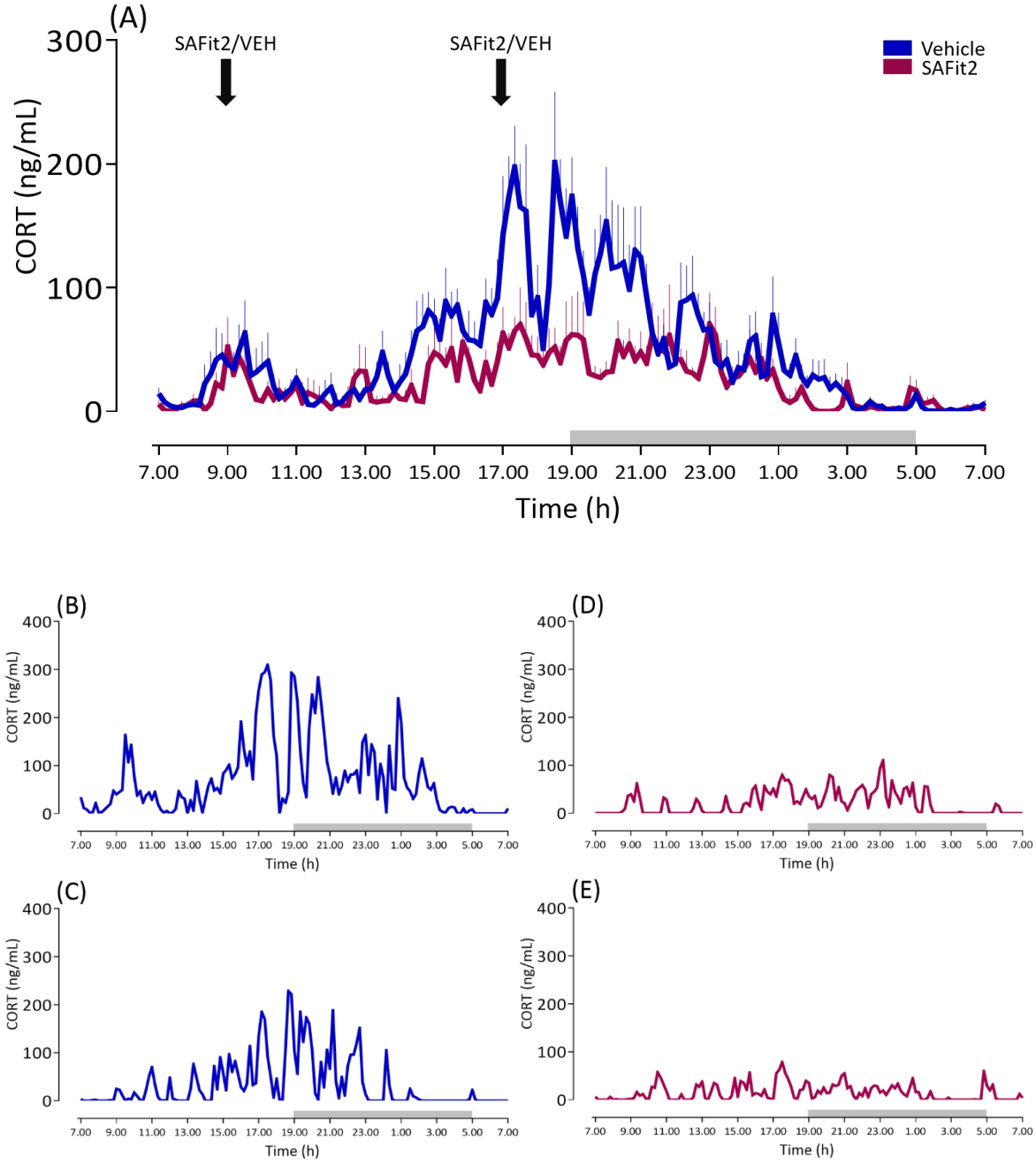

Figure 4.2. The effect of sub-chronic SAFit2 treatment on ultradian CORT secretion in male rats. *Mean + SEM CORT concentrations (A), and representative individual profiles (B-E) from male adult rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=7) or SAFit2 (20 mg/kg, s.c.; n=6) at 09.00h, and 17.00h for five consecutive days. Blood samples were collected via a cannula implanted in the right jugular vein every 10 min for 24-h on the fifth day, using an automated blood sampling system. Plasma corticosterone was measured by radioimmunoassay (RIA). Grey bar indicates light off (19:00-05:00h).*

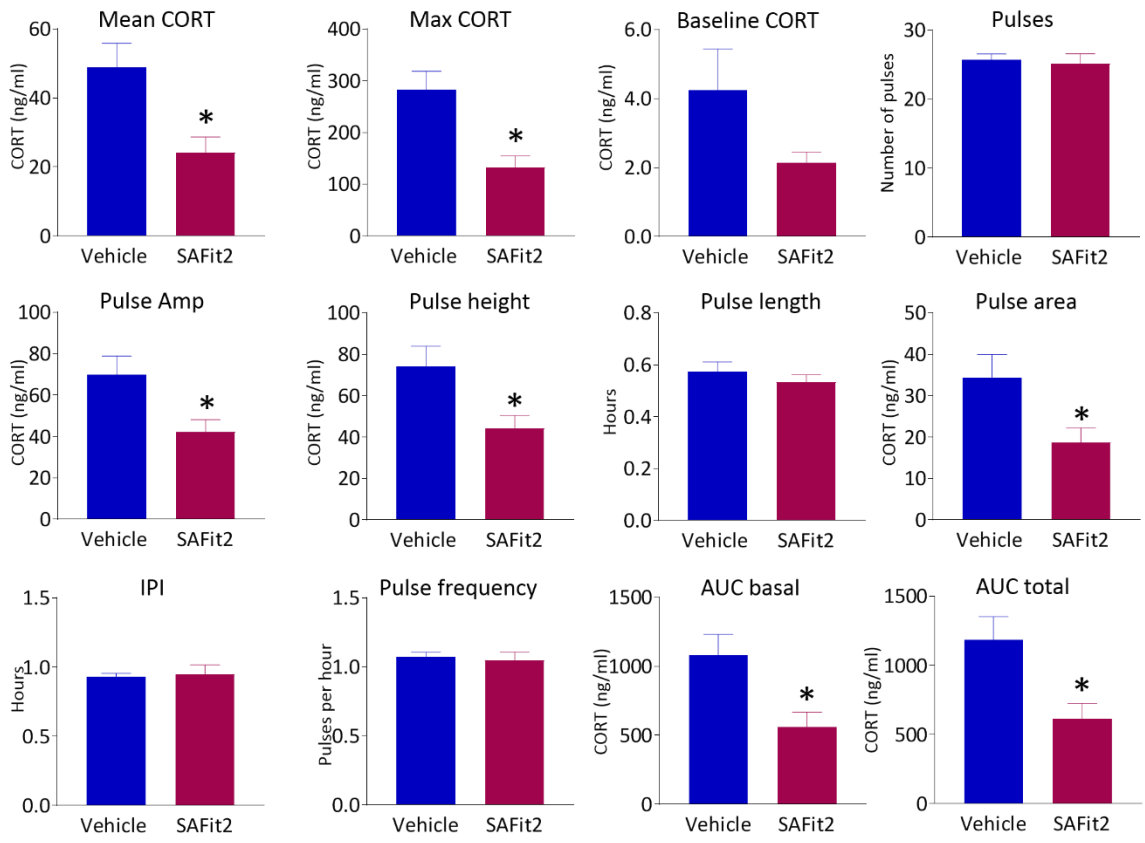

Figure 4.3. The effect of sub-chronic SAFit2 treatment on PULSAR parameters in male rats. *Mean + SEM CORT parameters calculated using PULSAR from 24-hour profiles of rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=7) or SAFit1 (20 mg/kg, s.c.; n=6) at 09.00h, and 17.00h for five consecutive days. Amp=amplitude, IPI=inter-pulse interval, AUC= area under the curve. Amp=amplitude, IPI= inter-pulse interval, AUC= area under the curve. *P<0.05 compared to vehicle-treated rats, with unpaired t-test.*

These results indicate that SAFit2 treatment in male rats decreases the overall CORT concentrations, as seen by decreased mean CORT in the SAFit2 group. Moreover, this effect is due to a decrease in amplitude of the ultradian pulses while the pulse length, IPI, and the number of pulses are unaffected. This suggests that central and peripheral inhibition of FKBP51 for a prolonged time result in decreased basal CORT concentration however there is no change in the dynamic of ultradian CORT pulsatility. Compared to acute SAFit2 administration in male rats, sub-chronic SAFit2 administration had more wide-ranging effects in reducing CORT concentrations under basal conditions.

4.3.2. The effect of sub-chronic SAFit1 treatment on basal CORT concentrations and ultradian rhythms in adult male rats

Even though there was no effect of acute SAFit1 on basal CORT secretion, the SAFit1 group showed decreased ACTH and CORT response to a CRH injection, suggesting that inhibition of peripheral FKBP5, presumably at the level of the corticotrophs in the anterior pituitary gland, does inhibit HPA activity. The hypothesis of this study was therefore that prolonged treatment would enhance the negative feedback in the pituitary gland sufficiently to achieve a decrease in basal CORT. Furthermore, the aim was to investigate whether a sub-chronic treatment with SAFit1 would affect the dynamics of ultradian pulsatility.

The same protocol as for the sub-chronic SAFit2 experiment was followed except for treatment frequency. As SAFit1 has a shorter half-life (2.5 hours compared to 9 hours for SAFit2; (347)), all animals in this experiment were treated with either vehicle or SAFit1 three times daily (compared to twice daily for SAFit2). Five vehicle-treated rats and four SAFit1-treated rats were treated for 5 consecutive days and on the fifth day they were all subject to ABS. Blood samples were taken every 10 minutes for 24 hours and processed for CORT concentrations. The mean was calculated for each time point for both groups and the average 24-h profiles are shown in figure 4.4 (A) along with the individual representative profile (B-E).

The individual 24-hour CORT profiles were separately analysed using PULSAR and the means for each parameter are shown in figure 4.5. There was a significant increase in the number of pulses ($t(7)=-2.60$, $P=0.035$), pulse frequency ($t(7)=-2.60$, $P=0.035$), and a significant decrease in the pulse length ($t(7)=-2.61$, $P=0.037$), the pulse area ($t(7)=2.25$, $P=0.032$), and the IPI ($t(7)=2.22$, $P=0.028$) in the SAFit1-treated rats compared to the vehicle-treated rats, as shown by an unpaired t-test. There was no significant effect of treatment on mean CORT ($t(7)=1.47$, $P=0.200$), max CORT ($t(7)=1.516$, $P=0.173$), basal CORT ($t(7)=0.131$, $P=0.900$), pulse amplitude ($t(7)=1.94$, $P=0.093$), pulse height ($t(7)=1.71$, $P=0.131$), AUC basal ($t(7)=1.75$, $P=0.123$) or AUC total ($t(7)=1.35$, $P=0.216$).

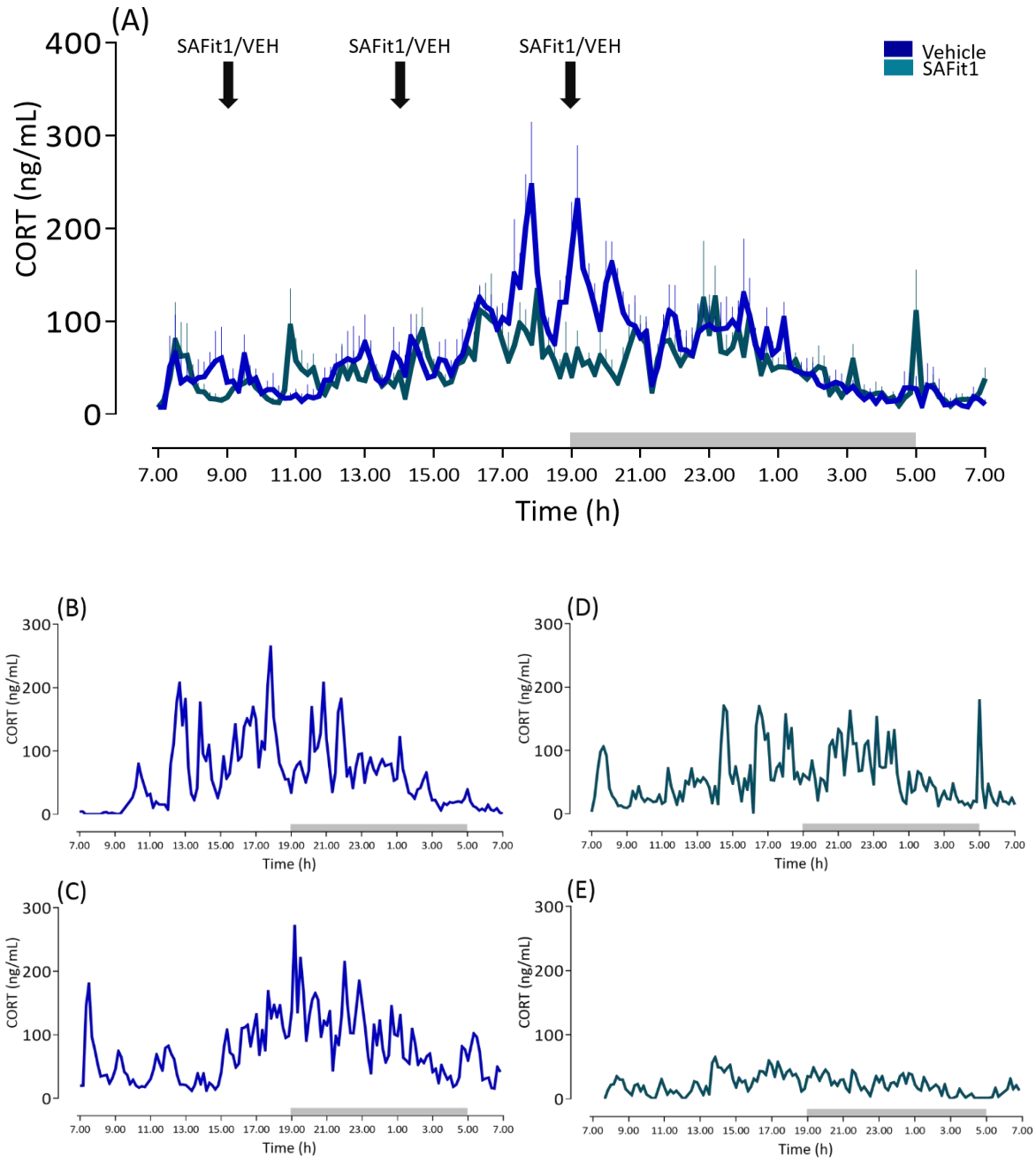

Figure 4.4. The effect of sub-chronic SAFit1 treatment on ultradian CORT secretion in male rats. *Mean + SEM (A) CORT concentrations, and representative individual profiles (B-E) from male adult rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=5) or SAFit2 (20 mg/kg, s.c.; n=4) at 09.00h, 14.00h, and 19.00h for five consecutive days. Blood samples were collected via a cannula implanted in the right jugular vein every 10 min for 24-h on the fifth day, using an automated blood sampling system. Plasma corticosterone was measured by radioimmunoassay (RIA). Grey bar indicates light off (19:00-05:00h).*

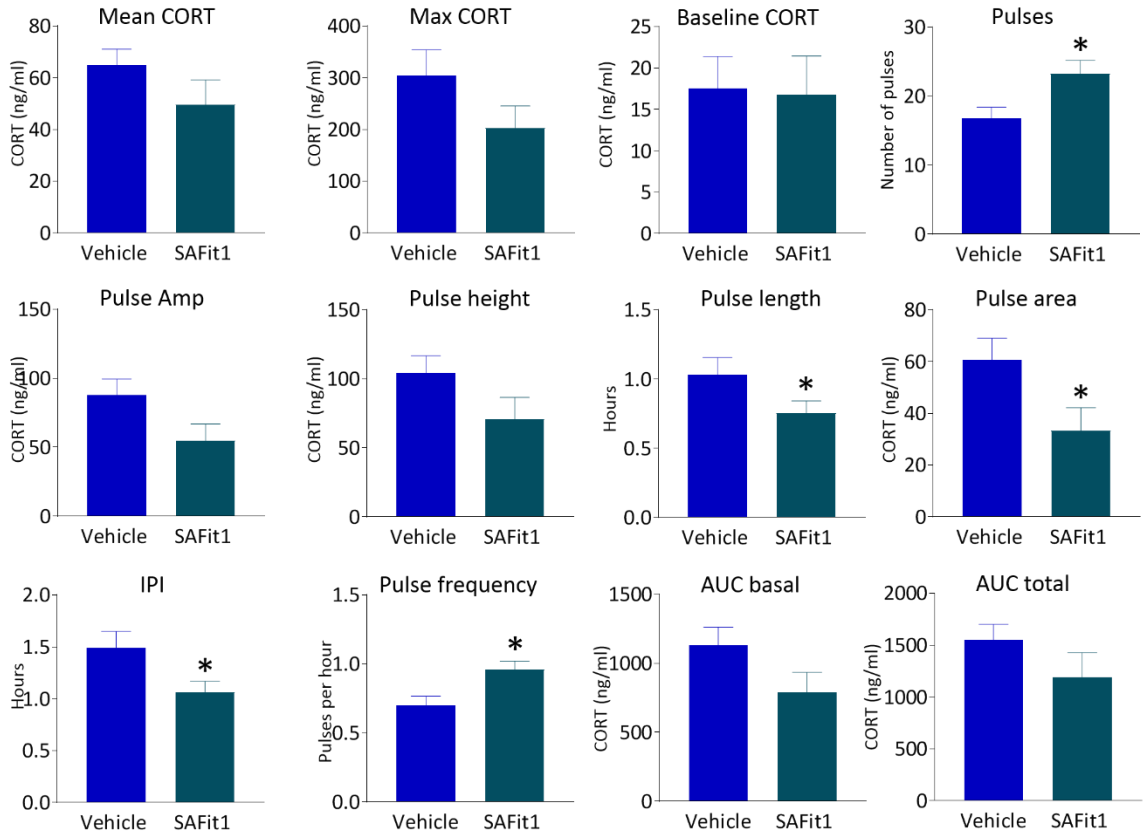

Figure 4.5. The effect of sub-chronic SAFit1 treatment on PULSAR parameters in male rats. *Mean + SEM CORT parameters calculated using PULSAR from 24-hour profiles of rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=5) or SAFit1 (20 mg/kg, s.c.; n=4) at 09.00h, and 17.00h. Amp=amplitude, IPI=inter-pulse interval, AUC= area under the curve. *P<0.05 compared to vehicle-treated rats, with unpaired t-test.*

These data indicate an effect of sub-chronic SAFit1 treatment on the dynamic of the pulse, rather than on the pulse amplitude and CORT concentrations. There is a higher number of pulses in the SAFit1 animals as seen by the increased number of pulses (23 pulses in the SAFit1-treated rats compared to 16 pulses in the vehicle-treated rats). The pulse length, pulse area, and IPI were both decreased in the SAFit1 animals. Interestingly there was no effect of SAFit1 treatment on mean CORT, basal CORT or total AUC. However, there was a trend towards decreased max CORT (P=0.059), pulse amplitude (P=0.080) and AUC basal (P=0.066) in the SAFit1-treated rats. This indicates that prolonged SAFit1 treatment affects the dynamic

of the ultradian CORT pulses, rather than CORT concentrations, as observed following SAFit2 treatment.

4.3.3. The effect of sub-chronic SAFit2 and SAFit1 on post-stress CORT concentrations in adult male rats

Following 24-h blood sampling and treatment with SAFit2 or SAFit1, the rats were subjected to noise stress on day 6 and blood sampling continued for 60 minutes following the noise stress (see schematics figure 4.1).

The mean + SEM CORT concentration at each time point was calculated for each group as shown in figure 4.6. In the SAFit2 experiment (figure 4.6. A), repeated measure ANOVA analysis showed an overall significant effect of time ($F(1.9, 15.0)=39.2$, $P=<0.00001$), a significant interaction between time and treatment ($F(1.9,15.0)=6.2$, $P=0.012$), and a significant effect of treatment ($F(1,8)= 6.92$, $P= 0.030$). Further post hoc analysis revealed a significant increase in stress-induced CORT in vehicle-treated rats at the 10 ($P=0.001$), 20 ($P<0.0001$), and 30 ($P<0.0001$) minute time points, compared to vehicle-treated rats at the 0 minute time point.

In comparison, no significant increase in stress-induced CORT in SAFit2-treated rats was observed at any time point. CORT was significantly lower in the SAFit2-treated rats at the 20 ($P=0.014$) and 30 minutes ($P=0.008$) time points compared to vehicle-treated rats at the same time points (figure 4.6 A). There was no difference between the baseline CORT at the 0 minute time point between vehicle-treated rats and SAFit2-treated rats.

These data indicate that sub-chronic SAFit2 treatment in male rats decreased the post-stress CORT concentrations sufficiently to diminish the stress response.

In the SAFit1 experiment (figure 4.6. B), repeated measures ANOVA analysis showed an overall significant effect of time ($F(2.4, 21.1)=22.7$, $P<0.000001$), a significant interaction between time and treatment ($F(2.4, 21.1)=3.9$, $P=0.031$), and a significant effect of treatment ($F(1,9)=6.4$, $P=0.032$). Post hoc analysis revealed a significant increase in stress-induced CORT in vehicle-treated rats at the 10 ($P<0.0001$), 20 ($P<0.0001$), and 30 ($P=0.001$) minutes time points, compared to vehicle-treated rats at the 0 minute time point. In comparison, no

significant increase in stress-induced CORT in SAFit1-treated rats was observed at any time point. There was a significant reduction in CORT in the SAFit1-treated rats at the 20 minutes (P=0.006) point compared to vehicle-treated rats at the respective time points. There was no difference between the baseline CORT at the 0 minute time point between vehicle-treated rats and SAFit1-treated rats.

Similar to the SAFit2-treated rats, the stress-mediated increase in CORT is diminished in the SAFit1-treated rats following 10 minutes of noise stress. These data demonstrate that, in contrast to acute treatment, prolonged treatment with a peripheral-only FKBP51 inhibitor can decrease the stress-induced CORT response.

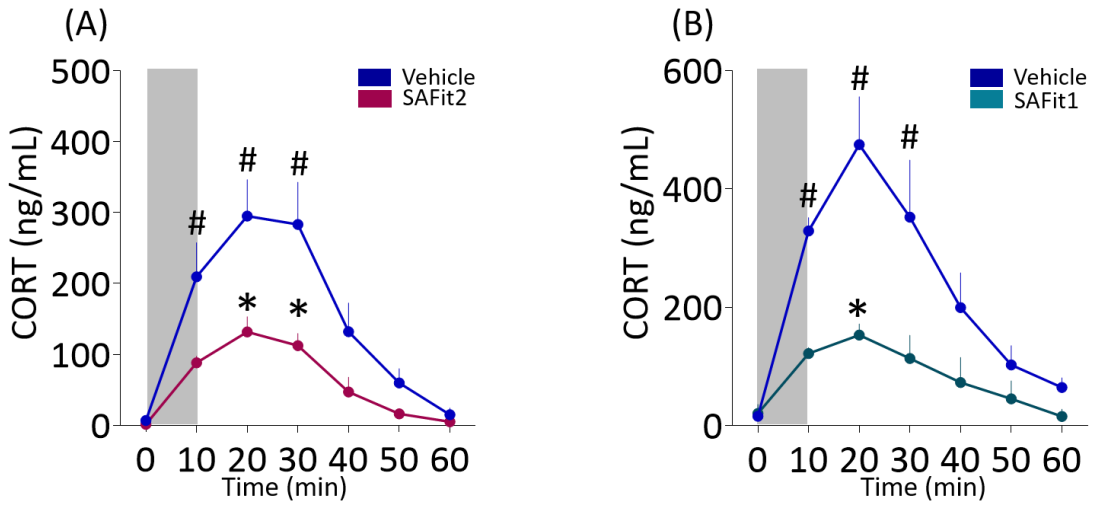

Figure 4.6. The effect of sub-chronic SAFit2 and SAFit1 treatment on stress-induced CORT secretion in male rats. *Sub-chronic treatment with either SAFit2 or SAFit1 both significantly lowered the noise-induced CORT secretion. (A) Mean + SEM corticosterone levels during a noise stress (96dB, 10 minutes, white noise, 07.00h) from male adult rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=5) or SAFit2 (20 mg/kg, s.c.; n=5) at 09.00h, and 17.00h for 5 consecutive days with the fifth day on the day before the noise stress. (B) Mean + SEM corticosterone levels during a noise stress (96dB, 10 minutes, white noise, 07.00h) from male adult rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=3) or SAFit1 (20 mg/kg, s.c.; n=3) at 09.00h, 14.00h, and 19.00h for 5 consecutive days with the fifth day on the day prior to the noise stress. Blood samples were taken every 10 minutes from a cannula implanted in the right jugular vein and CORT concentrations measured with CORT RIA. *P<0.05; compared to rats treated with vehicle at the same time point, with Tukey's post hoc test. #P<0.05; compared to vehicle-treated rats at time 0, with Tukey's post hoc test.*

4.3.4. The effects of sub-chronic SAFit2 and SAFit1 on gene expression following noise stress in adult male rats

In both experiments, the rats were sacrificed immediately following the collection of the last blood sample 60 minutes subsequent the noise stress. Tissues were dissected and extracted for RT-qPCR analysis for gene expression in the hypothalamus and anterior pituitary gland.

The effect of SAFit2 and SAFit1 treatment on HPA axis activity in the hypothalamus was investigated by quantifying the expression of the GR and MR, and CRH, AVP, and c-Fos. The c-Fos expression is a marker of general neuronal excitability, with an increased expression suggesting increased neuronal firing. To evaluate whether either compound has any effect of GR transcriptional activity, the expression of several GR-inducible genes, including FKBP5, GILZ, PER1, and SGK1 were quantified.

4.3.4.1. Hypothalamus

The relative expression of GR, MR, CRH, AVP, and c-Fos mRNA in the hypothalamus following the noise stress in the sub-chronic male SAFit2 experiment are shown in figure 4.7 A. An unpaired t-test revealed an effect of treatment on GR mRNA ($t(11)=-2.34$, $P=0.039$) with higher expression in the SAFit2-treated rats, and a trend towards higher c-Fos mRNA expression in the SAFit2-treated group was found ($t(11)=-1.88$, $P=0.086$). No significant effects of treatment on MR mRNA ($t(11)=-0.46$, $P=0.650$), CRH mRNA ($t(11)=-1.51$, $P=0.158$), or AVP mRNA ($t(11)=-0.31$, $P=0.741$) were found.

For the sub-chronic male SAFit1 experiment GR, MR, CRH, AVP, and c-Fos mRNA in the hypothalamus following the noise stress in are shown in figure 4.7 B. There was an effect of treatment on c-Fos mRNA ($t(10)=2.63$, $P=0.025$), with lower expression in the SAFit1-treated rats, as shown by unpaired t-test. There was no significant effect of treatment on GR mRNA ($t(10)=0.54$, $P=0.60$), MR mRNA ($t(10)=-0.46$, $P=0.651$), CRH mRNA ($t(10)=-0.42$, $P=0.678$), or AVP mRNA ($t(10)=0.34$, $P=0.741$).

Figure 4.7 C shows the hypothalamic mRNA expression of the GR-inducible genes, FKBP5, GILZ, PER1, and SGK1, in sub-chronically treated SAFit2 males following the noise stress. There was a significant effect of treatment on GILZ mRNA ($t(11)=-2.43$, $P=0.033$), and SGK1 mRNA ($t(11)=-2.44$, $P=0.033$), with increased expression in the SAFit2-treated rats. There was no significant effect of treatment on FKBP5 mRNA ($t(11)=-1.28$, $P=0.191$), or PER1 mRNA ($t(11)=-1.81$, $P=0.098$) as revealed by an unpaired t-test.

The mRNA expression following noise stress of the same GR-inducible genes from the sub-chronic SAFit1 male experiment is shown in figure 4.7 D. An unpaired t-test revealed no

effect of treatment on FKBP5 mRNA (t(10)=-0.41, P=0.690), GILZ mRNA (t(10)=0.04, P=0.965), PER1 (t(10)=1.22, P=0.250), and SGK1 mRNA (t(10)=1.59, P=0.141).

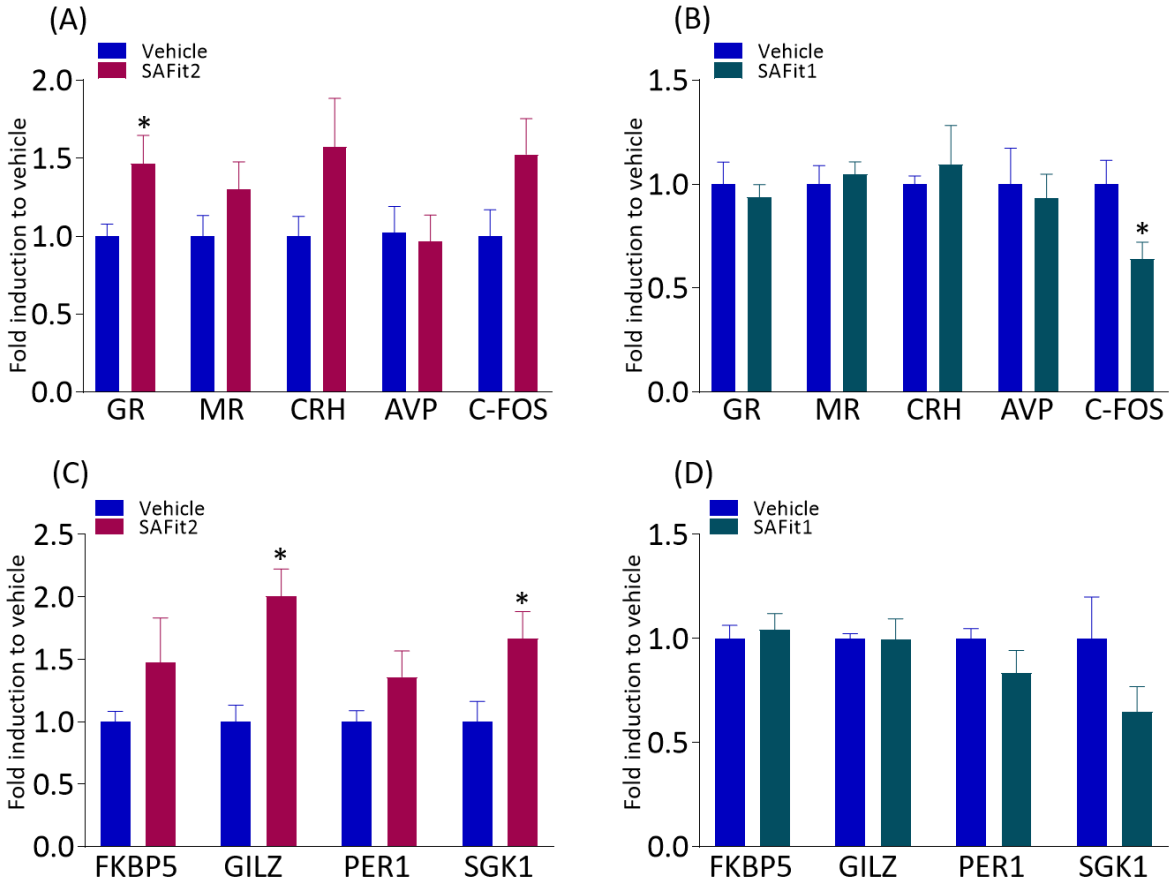

Figure 4.7. The effect of sub-chronic SAFit2 and SAFit1 treatment on stress-induced gene expression in the hypothalamus of male rats. *Mean + SEM expression of GR, MR, CRH, AVP, and c-Fos mRNA in the hypothalamus of male rats treated with Vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=5) or SAFit2 (20 mg/kg, s.c.; n=5) at 09.00h, and 17.00h for 5 consecutive days (A), and Vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=3) or SAFit1 (20 mg/kg, s.c.; n=3) at 09.00h, 14.00h, and 19.00h for 5 consecutive days (B). Mean + SEM expression of FKBP5, GILZ, PER1, and SGK1 mRNA in the hypothalamus of male rats treated with Vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=5) or SAFit2 (20 mg/kg, s.c.; n=5) at 09.00h, and 17.00h for 5 consecutive days (C), and Vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=3) or SAFit1 (20 mg/kg, s.c.; n=3) at 09.00h, 14.00h, and 19.00h for 5 consecutive days (D). *P<0.05 compared to vehicle, with unpaired t-test.*

4.3.4.2. Anterior pituitary gland

The expression of the same genes was characterised in the anterior pituitary gland and GR, MR, POMC, and c-Fos mRNA expression in the anterior pituitary gland following the noise stress in the sub-chronic male SAFit2 experiment is shown in figure 4.8 A. An unpaired t-test revealed a trend for an effect of treatment on c-Fos mRNA ($t(11)=-2.11$, $P=0.059$), with higher expression in the SAFit2 group. There was no effect of treatment on GR mRNA ($t(11)=-0.95$, $P=0.363$), MR mRNA ($t(11)=-1.31$, $P=0.216$), or POMC mRNA ($t(11)=-1.14$, $P=0.188$).

In the sub-chronic male SAFit1 experiment, an unpaired t-test revealed no effect of treatment on GR mRNA ($t(10)=0.89$, $P=0.391$), MR mRNA ($t(10)=0.22$, $P=0.827$), POMC mRNA ($t(10)=-1.73$, $P=0.113$), or c-Fos mRNA ($t(10)=0.25$, $P=0.804$), shown in figure 4.8 B.

The relative expression of FKBP5, GILZ, PER1, and SGK1 mRNA in the anterior pituitary gland following the noise stress in the sub-chronic male SAFit2 experiment is shown in figure 4.8 C. An unpaired t-test revealed a trend towards a significant effect of treatment on GILZ mRNA ($t(11)=-1.93$, $P=0.079$) with higher expression in SAFit2-treated rats. There was no significant effect of treatment on FKBP5 mRNA ($t(11)=-0.08$, $P=0.921$), PER1 mRNA ($t(11)=0.02$, $P=0.983$), and on SGK1 mRNA ($t(11)=-0.633$, $P=0.531$).

The same GR-inducible genes were measured in the anterior pituitary gland of the sub-chronic SAFit1 males, and are shown in figure 4.8 D. There was no effect of treatment on FKBP5 mRNA ($t(10)=-0.78$, $P=0.495$), GILZ mRNA ($t(10)=-0.05$, $P=0.954$), PER1 mRNA ($t(10)=0.43$, $P=0.672$), and SGK1 mRNA ($t(10)=0.007$, $P=0.995$), as revealed by an unpaired t-test.

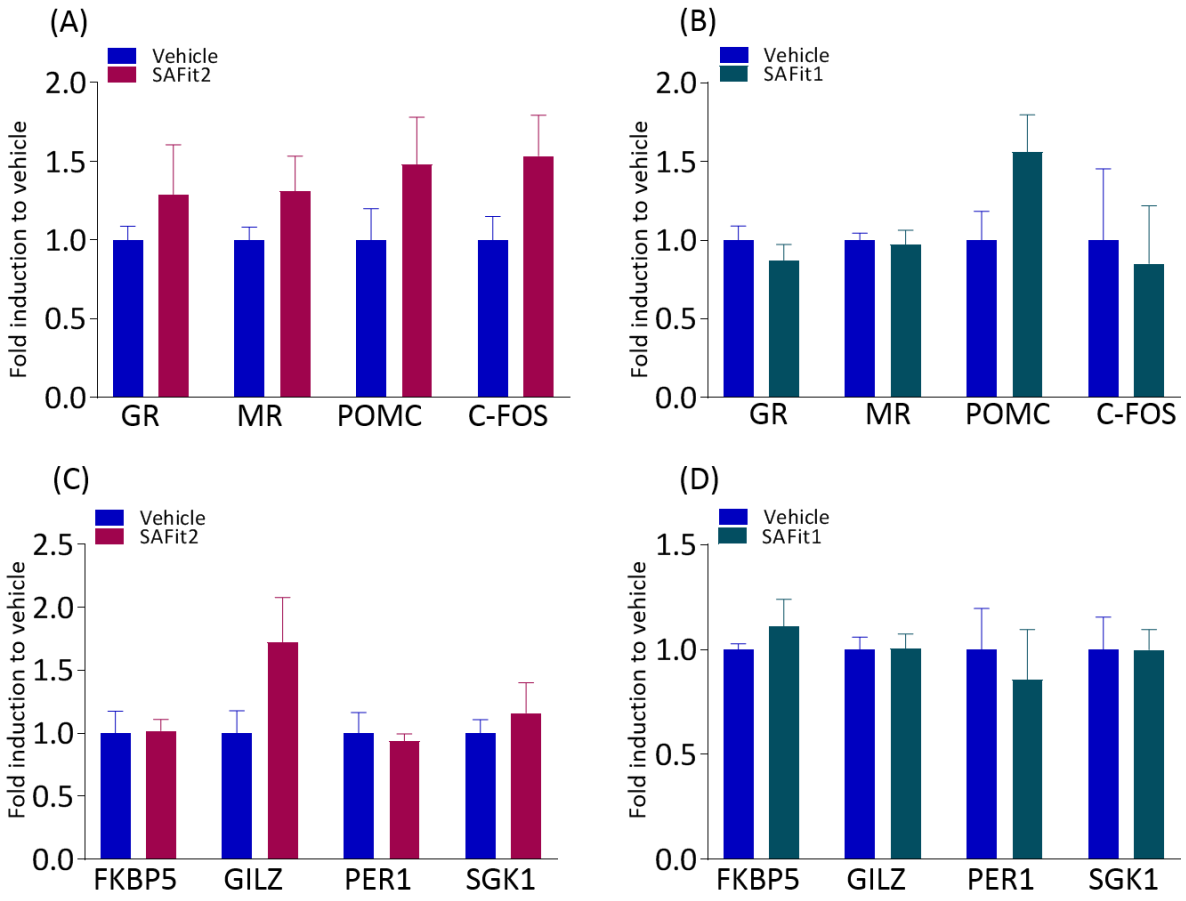

Figure 4.8. The effect of sub-chronic SAFit2 and SAFit1 treatment on stress-induced gene expression in the anterior pituitary gland of male rats. *Mean + SEM expression of GR, MR, CRH, AVP, and c-Fos mRNA in the anterior pituitary gland of male rats treated with Vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=5) or SAFit2 (20 mg/kg, s.c.; n=5) at 09.00h, and 17.00h for 5 consecutive days (A), and Vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=3) or SAFit1 (20 mg/kg, s.c.; n=3) at 09.00h, 14.00h, and 19.00h for 5 consecutive days (B). Mean + SEM expression of FKBP5, GILZ, PER1, and SGK1 mRNA in the in the anterior pituitary gland of male rats treated with Vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=5) or SAFit2 (20 mg/kg, s.c.; n=5) at 09.00h, and 17.00h for 5 consecutive days (C), and Vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=3) or SAFit1 (20 mg/kg, s.c.; n=3) at 09.00h, 14.00h, and 19.00h for 5 consecutive days (D). *P<0.05 compared to vehicle, with unpaired t-test*

4.4. Discussion

This chapter highlights the difference between the effects of SAFit2 and SAFit1 both on CORT pulsatility and on GR transcriptional activity. However, 5 days treatment with the central and peripheral FKBP51-inhibitor SAFit2, or the peripheral-only FKBP51-inhibitor, SAFit1, both result in lower stress-induced CORT secretion.

Sub-chronic SAFit2 resulted in decreased basal CORT, indicated by lower mean CORT, max CORT, pulse amplitude and height, and AUB. However, pulsatile dynamic analysis using the PULSAR algorithm revealed an effect of SAFit1 on pulsatility, with shorter pulses, shorter IPI and increased number of pulses in the SAFit1 group compared to vehicle-treated rats, an effect that was not seen in the SAFit2 group. This suggests that prolonged inhibition of FKBP51 in the periphery only causes a change in CORT ultradian pulsatility. In contrast, central, and peripheral inhibition of FKBP51 does not alter the CORT ultradian pulsatility however reduces CORT concentrations.

For decades it was thought that the CORT pulsatility was under hypothalamic control, and it is not until recently that a model of a pituitary-adrenal gland feed-forward/feedback loop was suggested to produce the ultradian pulses (130, 132). Walker and colleagues showed that pulsatile CORT secretion occurs without a pulsatile CRH input to the pituitary gland (130). For example, a constant infusion of a physiological dose of CRH results in pulses of ACTH, followed by pulses of CORT (131). In contrast, inhibiting the SCN output disrupts the circadian rhythmicity of CORT secretion however the ultradian CORT pulses remain intact (131).

Interestingly, the pulse-generator model proposed by Walker and colleagues (131, 132) suggests that if the adrenal gland delay is reduced (produced by decreased ACTH) and the CRH drive remains constant, this will produce shorter CORT pulses. This is consistent with the result seen in the SAFit1 experiment. The model also predicts that a decrease in adrenal gland delay and a simultaneous decrease in CRH drive would cause no difference in the CORT pulse length. This is consistent with the results of the SAFit2 experiment. The exact mechanism of ultradian CORT pulsatility is yet to be unravelled however this study is the first, to my knowledge, to show that altering GR activity in the periphery only (with SAFit1), increased the ultradian CORT pulse frequency (figure 4.3. and 4.4.). This is an important step

in gaining further knowledge in the regulation, and hence the importance of ultradian CORT pulses and their regulation.

What is the physiological relevance of CORT pulsatility? As both ACTH and CORT are released in pulsatile manners, the two cognate receptors are exposed to their ligands in oscillatory signals and these oscillations of ligands are important for a rapid response in the target tissues. Indeed, an *in vivo* study in the rat investigated the effect of constant versus pulsatile ACTH infusion on the CORT response and showed that only the pulsatile ACTH infusion elicited a CORT response (384). Similarly, *in vitro* studies in AtT-20 cells indicates that a pulse of CORT activates intracellular GR in a rapid and transient manner (385).

Physiological conditions such as ageing and certain disease states produce an alteration in this ultradian CORT pulsatility (302). Cushing's syndrome refers to adrenal gland hyperactivity irrespective of aetiology (386), whilst Cushing's disease is a secondary hypercortisolism caused by overproduction of ACTH from the anterior pituitary gland, often as a result of a pituitary gland tumour (387). In Cushing's disease the majority of patients exhibit a blunted circadian cortisol secretion, however, in a third of patients there is an increase in the magnitude of the ultradian pulses (302). Similarly, patients with primary adrenal gland Cushing's syndrome exhibit overall increased cortisol concentrations throughout the day, and this is produced by an increase in amplitude and frequency of the ultradian cortisol pulses (388). Furthermore, patients suffering from melancholic depression have overall increased circulating cortisol as a result of the increased amplitude of the ultradian pulses throughout the day (300). It is not yet understood how these disease states produce these alterations to the ultradian pulsatility however it is thought that a dysfunctional GC negative feedback mechanism is involved. The changes in ultradian pulse frequency following sub-chronic SAFit1 administration (figure 4.3. and 4.4.) is possibly the first study to date to show that changes in peripheral GR activity, and hence changes in the pituitary-adrenal gland pulse generator, can alter the frequency of ultradian CORT pulses. The increase in pulse frequency in the SAFit1-treated animals is likely to be due to a lower ACTH secretion from the pituitary gland caused by enhanced GR-mediated negative feedback (section 3.3.5), although the molecular mechanism is yet to be unravelled.

In this chapter, it was also demonstrated that sub-chronic SAFit2 treatment induced an increase in GR transcriptional activity as seen by increased expression of the GR-responsive

genes GILZ and SGK1 in the hypothalamus. There was no effect of SAFit1 treatment on any of the GR-inducible genes measured and this is not surprising as the SAFit1 should not be active centrally. In the hypothalamus, the c-Fos mRNA expression increased following noise stress in the SAFit2 group and decreased in the SAFit1 group compared to respective vehicle-treated rats. As c-Fos in the brain can be used as a marker of general neuronal activity (378), this suggests an increase in neuronal activity in the hypothalamus following SAFit2 treatment and a decrease in neuronal activity in the hypothalamus following SAFit1 treatment.

Although SAFit1 does not cross the BBB, this effect is rather surprising and could be due to a decrease in neuronal activity unrelated to the effects of SAFit1. As c-Fos expression can be used as a marker of general neuronal expression (378), this suggests that prolonged SAFit2 treatment increases the neuronal activity in the hypothalamus following the noise stress. It is difficult to determine what exact neuronal activity is enhanced, however, it is tempting to speculate that it is related to the GC-mediated negative feedback to reduce the stress-induced CORT in the SAFit2-treated rats. It is likely that the decrease in c-Fos in the SAFit1-treated rats, as the SAFit1 does not reach the brain, is secondary to the effects of SAFit1 in the periphery. Hence this effect would be present in the SAFit2-treated rats too, and the overall increase in c-Fos expression is specific to the effects of SAFit2.

Further, the results show an increased GR transcriptional activity in the hypothalamus following the SAFit2 treatment, despite a lower secretion of CORT following stress in this group, whilst there was no effect of the SAFit1 treatment. This indicates that sub-chronic SAFit2 treatment enhances GR transcriptional activity, and it is plausible that this is by increasing nuclear GR as a result of less FKBP51-bound GRs, caused by the SAFit2. In comparison, the SAFit1 does not enter the brain and thus is it not surprising that there was no increase in those GR-inducible genes.

The expression of GR is regulated by various pathways, including cAMP and autoregulation where GR can bind to a GRE within its gene (376, 377). Indeed, it has been shown that GCs, such as DEX, can downregulate GR expression (375). Thus, perhaps GR expression is increased following sub-chronic SAFit2 administration as a result of decreased circulating levels of CORT.

These data indicate a small increase in GR transcriptional activity in the hypothalamus following SAFit2 treatment at 60 minutes post-noise stress. This effect was not observed in the SAFit1 group. The expression of these GR-inducible genes was measured 60 minutes following noise stress and the expression profiles are likely different under basal conditions. Nevertheless, these results indicate that there is no enhancement in transcriptional GR activity following SAFit1, and a slight enhancement following SAFit2. Interestingly, both SAFit2 and SAFit1 resulted in lower stress-induced CORT increase following sub-chronic treatment. It is plausible that SAFit2 causes this reduction in CORT by the enhanced transcriptional GR activity, as a result of increased nuclear GR. This could enhance GR binding the nGRE in the POMC gene and reduce ACTH concentrations, and reduce CORT secretion. Although there was no reduction in POMC mRNA expression observed in this study only one time point was used and the whole anterior pituitary gland for measurements. Perhaps additional time points and a more anatomically sensitive technique would reveal differences in POMC expression. Moreover, studies suggest that GR can interact with CREB in the PVN to prevent CREB binding to the CRE on the CRH promoter (194, 195). Thus, it is plausible that increased nuclear GR will result in reduced CRH and subsequently CORT, and perhaps this is the mechanism by which SAFit2 reduces the CORT concentrations. Although there was no difference in CRH mRNA expression in the SAFit2-treated animals compared to the vehicle-treated animals, the expression was measured in whole hypothalamic extract and therefore does not only include the PVN but also other nuclei. The CRH mRNA expression was measured at one time point only, and given that changes in CRH expression in response to stress are highly dynamic (389) this may not be the time point at which the difference is most apparent. In contrast, there was no effect of sub-chronic SAFit1 treatment on the GR-inducible genes measured in this experiment. This suggests that perhaps the effects of SAFit1 are due to non-genomic GR activity. It is also possible that the peak expression of the GR-inducible genes was missed in this experiment.

Perhaps a more suitable technique for measuring gene expression would have been ISH which allows for anatomical quantification of the gene expression. Alternatively, punching out specific areas, such as the PVN, from brain slices and performing RT-qPCR would have allowed for a more specific gene expression analysis. More time points would have allowed

for a timeline of gene expression following stress however, as previously mentioned, the limited availability of the SAFit compounds meant this was not possible.

Further, neither SAFit2 nor SAFit2 caused induction of any of the GR-inducible genes measured in the anterior pituitary gland. Since SAFit1 is only active peripherally, this indicates that either the peak of the transcriptional activity of these GR-inducible genes are at a different time point, or that the actions of SAFit1 at the pituitary gland are non-genomic.

In this chapter, evidence for altered pulsatility following peripheral-only FKBP51 inhibition is presented. There has been a long-standing notion that ultradian CORT pulsatility is centrally regulated, however, recent evidence suggests a system comprising a pituitary-adrenal gland feed-forward/feedback loop to regulate CORT pulsatility (131, 132). The data in this chapter support this model and highlights the important role of the pituitary-adrenal gland system in the regulation of HPA axis pulsatility.

Chapter 5: Treatment with FKBP51 inhibitors in female rats

5.1. Introduction

Substantial evidence indicates sexual dimorphism in the HPA axis, both in humans and in rodents. Female rats have higher overall basal and stress-induced CORT concentrations (reviewed (231)), and ovariectomized females show similar CORT concentrations as sham males (239). This suggests a strong role of sex steroids in influencing HPA axis activity. Global FKBP51-knock out in female mice had a stronger effect than in male mice, with reduced basal and stress-induced CORT concentrations, whereas there was no effect on basal CORT concentrations in male 51KO (342, 343). This evidence suggests that perhaps FKBP51 has a more prominent role in regulating HPA axis activity in female mice.

Therefore, in this chapter data showing the effects of SAFit2 and SAFit1 administration in female adult rats on both basal and stress-induced CORT secretion will be presented. Furthermore, the expression of GR, MR, and FKBP51 in male and female control animals will be shown, and the effect of SAFit2 and SAFit1 on gene expression following noise stress will be discussed.

5.2. Methods

All the female rats used for the experiments in this chapter were freely cycling Sprague-Dawley and no tests for the stage of the oestrus cycle were performed.

5.2.1. Experiments 9 and 10: Effect of acute SAFit2 and SAFit1 treatment on CORT ultradian rhythm and stress-induced CORT secretion in intact female rats.

For the SAFit2 experiments rats were treated with either vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in 0.09% sterile saline; 2 ml/kg; s.c. n=7) or SAFit2 (20 mg/kg; s.c.; n=7) at 09.00h and 17.00h on the day of blood sampling. For SAFit1 experiments, rats were treated with either vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in 0.09% saline; 2 ml/kg; s.c. n=9) or SAFit1 (20 mg/kg; s.c.; n=3) at 09.00h, 14.00h and 19.00h on the day of blood sampling. Note that this experiment protocol differed from the SAFit2 experiment only in the times for the administration of the SAFit compounds, due to a shorter half-life of the SAFit1

this compound was administered three times compared to two times for the SAFit2 (figure 5.1.).

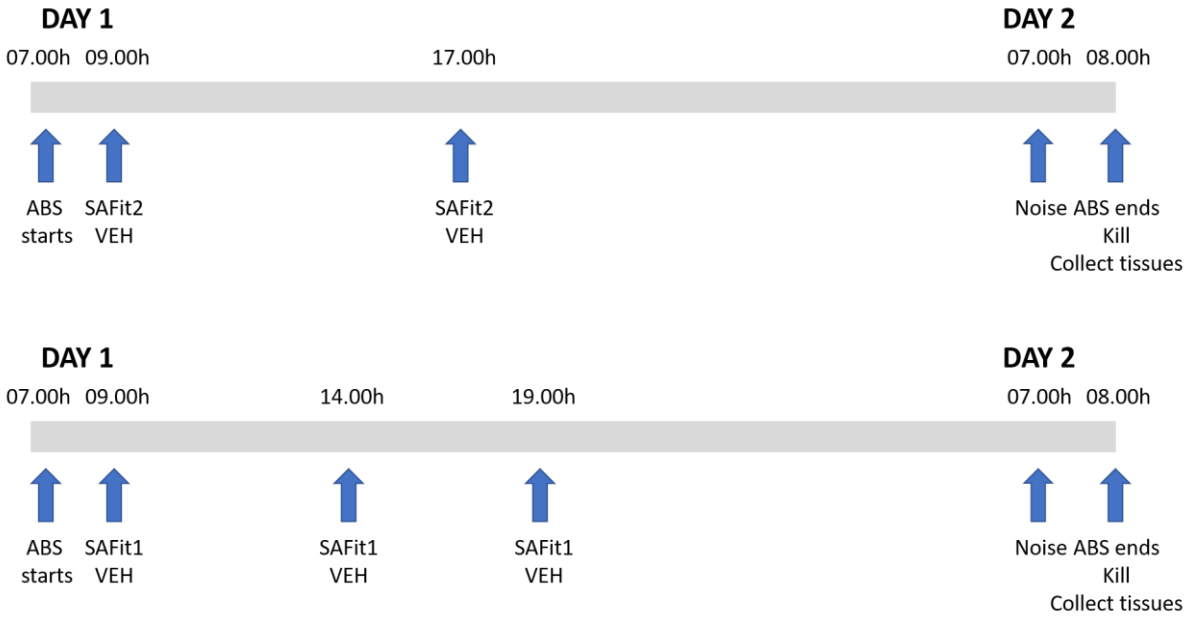

Figure 5.1. Experimental schedule for the acute SAFit2 and SAFit1 treatment in female rats.

5.2.2. Experiment 11: Effect of sub-chronic SAFit2 treatment on CORT ultradian rhythm and stress-induced CORT secretion in female rats

For SAFit2 experiments rats were treated with either vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in 0.09% sterile saline; 2 ml/kg; s.c., n=7) or SAFit2 (20 mg/kg; s.c.; n=6) at 09.00h and 17.00h for 5 consecutive days, with the fifth day on the day of blood sampling.

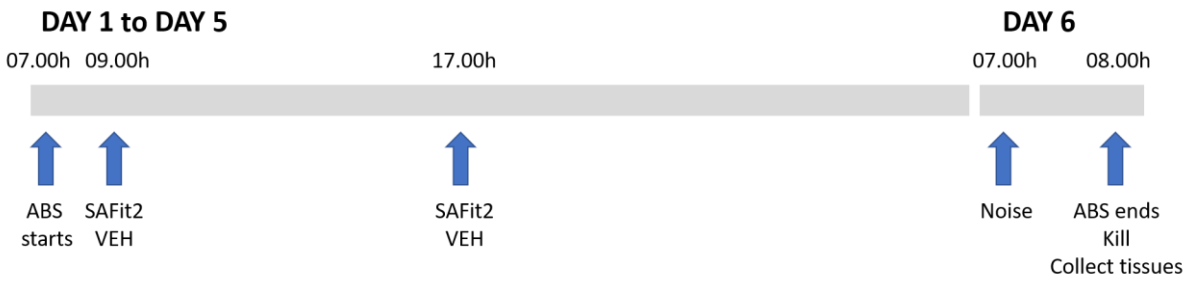

Figure 5.2. Experimental schedule for the sub-chronic SAFit2 treatment in female rats.

For all three SAFit1 and SAFit2 experiments, ABS for measurement of basal CORT was conducted from 07.00h on day 1, or day 5, until 07.00h the following day. At 07.00h of day 6, all rats were subject to noise stress (10 min, 96dB, white noise) and blood sampling collection continued for one more hour. Immediately after the sampling had stopped at 08.00h the rats were euthanized using pentobarbitone injection in the i.v. cannula followed by decapitation with a guillotine. The brains were dissected to isolate specific areas, and, along with the anterior pituitary gland and adrenal gland, were snap-frozen on dry ice to be used for further gene expression analysis.

Due to time restraints, for the SAFit1 experiment, the data from the vehicle group was pooled with the vehicle group from the acute SAFit2 female experiment for all data analysis. The analysis for the SAFit2-treated rats was only compared to the respective vehicle-treated animals, not with the pooled vehicle-treated animals.

5.2.3. Experiment 12: Expression of FKBP5, GR and MR in the brain and anterior pituitary gland of intact male and female rats

Female and male Sprague-Dawley of 8 weeks old were euthanized in the AM (08.00h-09.00h) or PM (16.30h-17.30h) using isoflurane. Brains and anterior pituitary gland were dissected and snap-frozen on dry ice and further processed for gene expression analysis. The groups comprised: male AM (n=6), male PM (n=6), female AM (n=6), and female PM (n=6).

5.3. Results

5.3.1. The effect of acute SAFit2 treatment on basal CORT concentrations and ultradian rhythms in adult female rats

In this experiment, the same protocol was followed as for the acute SAFit2 ABS experiment in male rats. Seven vehicle-treated rats and seven SAFit2-treated rats were all subject to ABS every 10 minutes for 24 hours on the same day as treatment. The mean 24-h CORT profiles are shown in figure 5.3 (A) along with the individual representative profile (B-E).

To allow for a quantitative comparison of the CORT parameters, the individual 24-hour CORT profiles were analysed using PULSAR. The means for each parameter are shown in figure 5.4. An unpaired t-test revealed no significant effect of treatment on any of the parameters tested, including mean CORT ($t(11)=-0.66$, $P=0.522$), max CORT ($t(11)=0.91$, $P=0.385$), baseline CORT ($t(11)=-0.95$, $P=0.358$), number of pulses ($t(11)=1.78$, $P=0.103$), pulse amplitude ($t(11)=0.12$, $P=0.905$), pulse height ($t(11)=-0.03$, $P=0.976$), pulse length ($t(11)=-1.27$, $P=0.227$), pulse area ($t(11)=-1.23$, $P=0.242$), IPI ($t(11)=-1.14$, $P=0.275$), pulse frequency ($t(11)=1.61$, $P=0.125$), AUC basal ($t(11)=-0.39$, $P=0.698$), or AUC total ($t(11)=-0.61$, $P=0.555$).

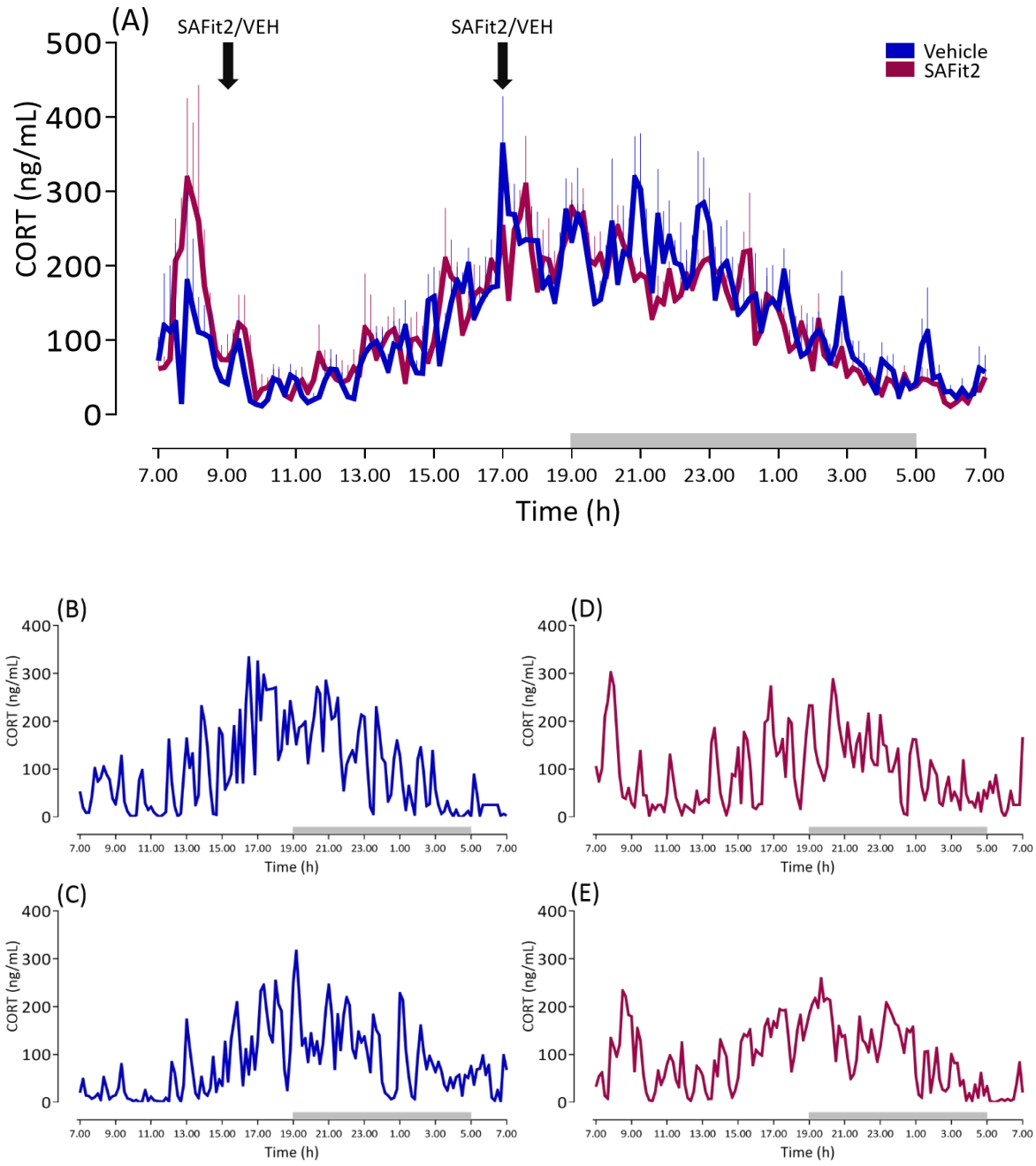

Figure 5.3. The effect of acute SAFit2 treatment on ultradian CORT secretion in female rats. *Mean + SEM (A) CORT concentrations, and representative individual profiles (B-E) from female adult rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=7) or SAFit2 (20 mg/kg, s.c.; n=7) or at 09.00h, and 17.00h. Blood samples were collected via a cannula implanted in the right jugular vein every 10 min for 24-h using an automated blood sampling system. Plasma corticosterone was measured by radioimmunoassay (RIA). Grey bar indicates light off (19:00-05:00h).*

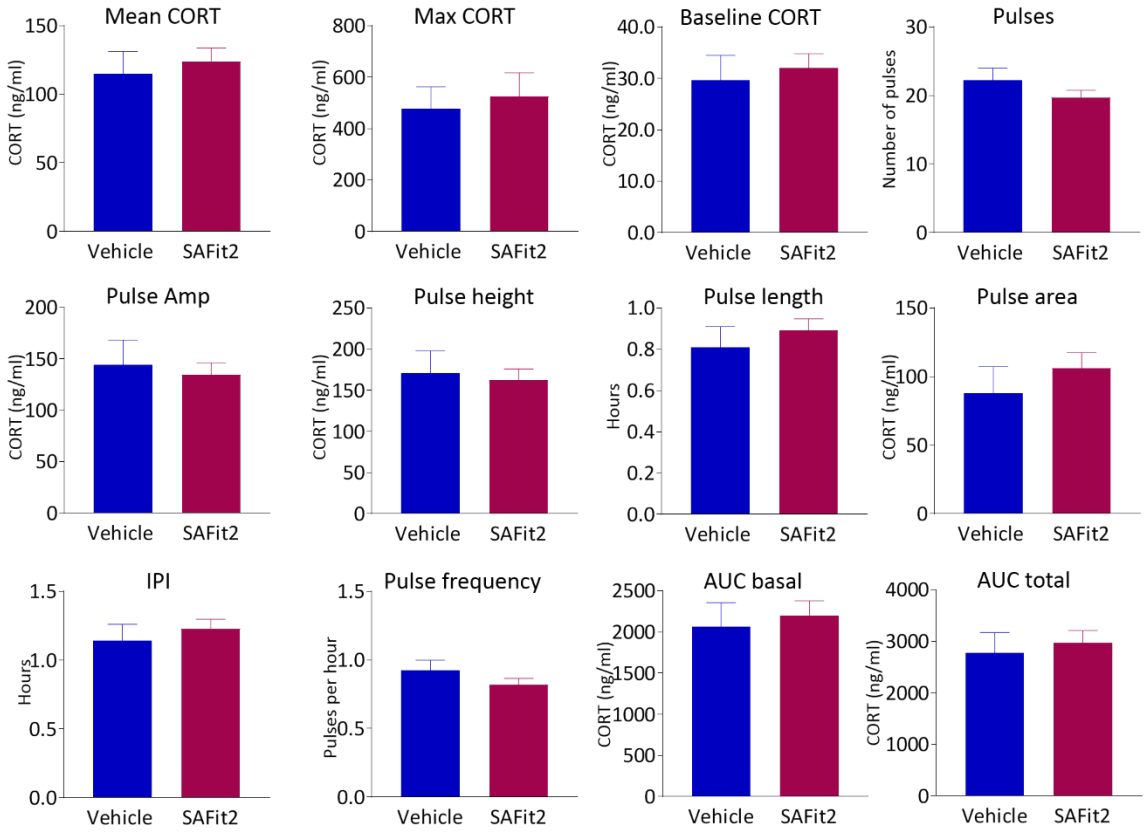

Figure 5.4. The effect of acute SAFit2 treatment on PULSAR parameters in female rats. *Mean + SEM CORT parameters calculated using PULSAR from 24-hour profiles of rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; n=5) or SAFit1 (20 mg/kg, s.c.; n=7) at 09.00h, and 17.00h. Amp=amplitude, IPI=inter-pulse interval, AUC= area under the curve. Amp=amplitude, IPI= inter-pulse interval, AUC= area under the curve.*

These data are in contrast with the data showing an effect of acute SAFit2 in male rats, with significantly lower maximum CORT concentrations. This highlights an important difference in the regulation of basal HPA axis activity in males and females following the inhibition of FKBP51. Nevertheless, a higher dose of, or prolonged treatment with SAFit2 may be required to observe a significant reduction of CORT secretion in female rats.

5.3.2. The effect of SAFit1 treatment on basal CORT concentrations and ultradian rhythms in adult female rats

To further investigate the effect of FKBP51 inhibition in female rats and the role of FKBP51 in HPA axis sexual dimorphism, SAFit1 was administered acutely in female rats and the effect on basal HPA axis activity was investigated. Since there was no effect of acute SAFit2 administration on basal CORT concentrations or the ultradian pulsatile pattern, it would be interesting to investigate whether a peripheral-only FKPBP51 inhibition would have different effects. As already mentioned, the use of SAFit1 allows the distinguishing between central and peripheral mechanisms, and to investigate what effect may occur when peripheral-only FKBP51 inhibition is achieved. Furthermore, as acute SAFit1 administration in male rats (chapter 3) did not have any effects on basal CORT concentration or CORT pulsatility, this experiment allowed the comparison of a potential sex difference in the peripheral regulation of CORT secretion.

The same sampling protocol was followed as for the acute female SAFit2 ABS experiment, and the acute male SAFit2 and SAFit1 ABS experiments, and the animals were treated at 09.00h, 14.00h, and 19.00h on the same day as sampling.

Twenty-four-hour sampling profiles of CORT concentrations were obtained for three vehicle-treated and five SAFit1-treated animals and the mean average CORT concentration was calculated for each time point. Individual representatives and the resultant mean profiles are shown in fig 5.5. Due to technical problems with the sampling procedure (i.e. lack of cannula patency), and time limitations, full 24-h CORT profiles from only three vehicle-treated rats were obtained, and therefore the data was pooled with the vehicle-treated rats from the female acute SAFit2 experiment. Although this is not ideal due to difference in treatment frequency and timings, and different batches of animals, an unpaired t-test of the two vehicle-treated groups did not reveal any significant differences for any of the PULSAR parameters, suggesting that the two vehicle-treated groups are comparable.

All the individual 24-hour profiles were analysed with PULSAR and the means for each parameter are shown in figure 5.6. Further, an unpaired t-test revealed a significant effect of treatment on mean CORT ($t(10)=2.48$, $P=0.033$), AUC basal ($t(10)=2.89$, $P=0.016$) and AUC total ($t(10)=2.36$, $P=0.039$). In addition, there was a trend of lower pulse amplitude

(t(10)=2.05, P=0.067) and pulse height (t(10)=1.95, P=0.080), with all parameters decreased in the SAFit1-treated rats compared to vehicle-treated rats. There was no effect of treatment on max CORT (t(10)=1.00, P=0.340), baseline CORT (t(10)=0.91, P=0.379), number of pulses (t(10)=-0.26, P=0.795), pulse length (t(10)=0.034, P=0.974), pulse area (t(10)=0.27, P=0.790), IPI (t(10)=0.51, P=0.625), or pulse frequency (t(10)=-0.26, P=0.795).

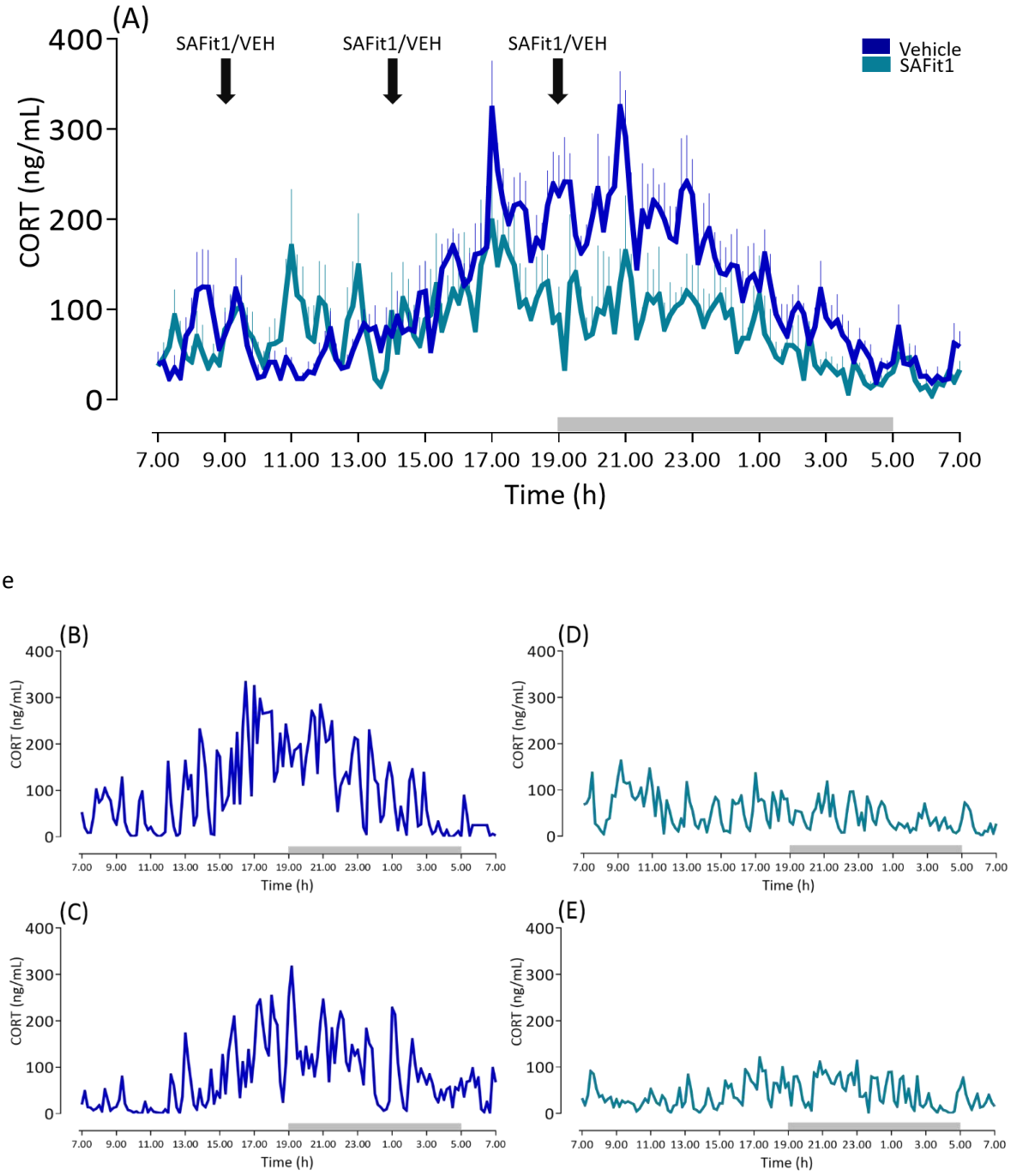

Figure 5.5. The effect of acute SAFit1 treatment on ultradian CORT secretion in female rats. *Mean + SEM (A) CORT concentrations, and representative individual profiles (B-E) from female adult rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=3+6) or SAFit1 (20 mg/kg, s.c.; n=4) or at 09.00h, 14.00h, and 19.00h. Blood samples were collected via a cannula implanted in the right jugular vein every 10 min for 24-h using an automated blood sampling system. Plasma corticosterone was measured by radioimmunoassay (RIA). Grey bar indicates light off (19:00-05:00h).*

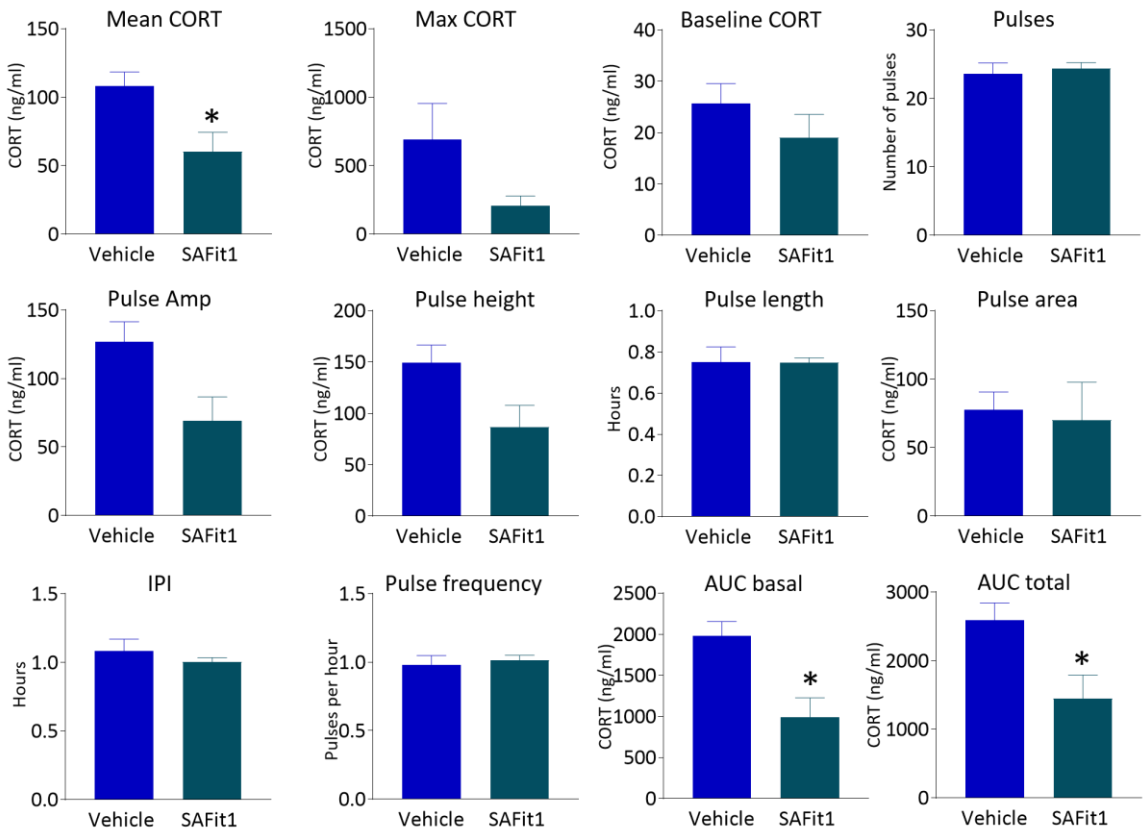

Figure 5.6. The effect of acute SAFit1 treatment on PULSAR parameters in female rats. *Mean + SEM CORT parameters calculated using PULSAR from 24-hour profiles of rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=3+6) or SAFit1 (20 mg/kg, s.c.; n=4) at 09.00h, and 17.00h. Amp=amplitude, IPI=inter-pulse interval, AUC= area under the curve. Amp=amplitude, IPI= inter-pulse interval, AUC= area under the curve. *P<0.05 compared to vehicle-treated rats, with unpaired t-test.*

5.3.3. The effect of acute SAFit2 and SAFit1 treatment on post-stress CORT concentrations in adult female rats

Following 24-h blood sampling and treatment with SAFit2 or SAFit1, the rats were subjected to noise stress and blood sampling continued for 60 minutes following the noise stress (see schematic figure 5.1).

The mean + SEM CORT concentration at each time point was calculated for each group as shown in figure 5.7. In the SAFit2 experiment (figure 5.7 A), repeated measures ANOVA analysis showed a significant effect of time ($F(2.1,24.8)=96.2$, $P=<0.00001$), no significant

interaction between time and treatment ($F(2.1,24.8)=2.0$, $P=0.146$), and only a trend toward a significant effect of the treatment was found ($F(1,12)=4.193$, $P=0.063$). Further, post hoc analysis revealed a significant increase in stress-induced CORT in vehicle-treated rats at the 10 ($P<0.0001$), 20 ($P<0.0001$), and 30 ($P<0.0001$) minute time points, compared to vehicle-treated rats at the 0 minute time point. A significant increase in stress-induced CORT in SAFit2-treated rats was also observed at the 10 ($P<0.0001$), 20 ($P<0.0001$), and 30 ($P<0.0001$) minute time points, compared to SAFit2-treated rats at the 0 minute time point. There was no difference between the baseline CORT at the 0 minute time point between vehicle-treated rats and SAFit2-treated rats.

These data suggest there is no difference in stress-induced CORT secretion in SAFit2-treated rats compared to vehicle-treated rats.

In the SAFit1 experiment (figure 5.7 B), repeated measures ANOVA analysis showed a significant effect of time ($F(2.5,32.2)=45.7$, $P=<0.00001$), a significant interaction between time and treatment ($F(2.5,32.2)=4.9$, $P=0.009$), and a significant effect of treatment ($F(1,13)=9.003$, $P=0.01$). Further, post hoc analysis revealed a significant increase in stress-induced CORT in vehicle-treated rats at the 10 ($P<0.0001$), 20 ($P<0.0001$), and 30 ($P<0.0001$) minutes time points, compared to vehicle-treated rats at the 0 minute time point. In comparison, no significant increase in stress-induced CORT in SAFit1-treated rats was observed at any time points. Plasma CORT concentrations were significantly lower in the SAFit1-treated rats at the 10 minutes ($P=0.005$), 20 minutes ($P=0.001$) and 30 minutes ($P=0.005$) time points compared to vehicle-treated rats at respective time points. There was no difference between the baseline CORT at the 0 minute time point between vehicle-treated rats and SAFit2-treated rats.

These data indicate that, compared to vehicle-treated rats, acute SAFit1 treatment in female rats results in lower post-stress CORT concentrations.

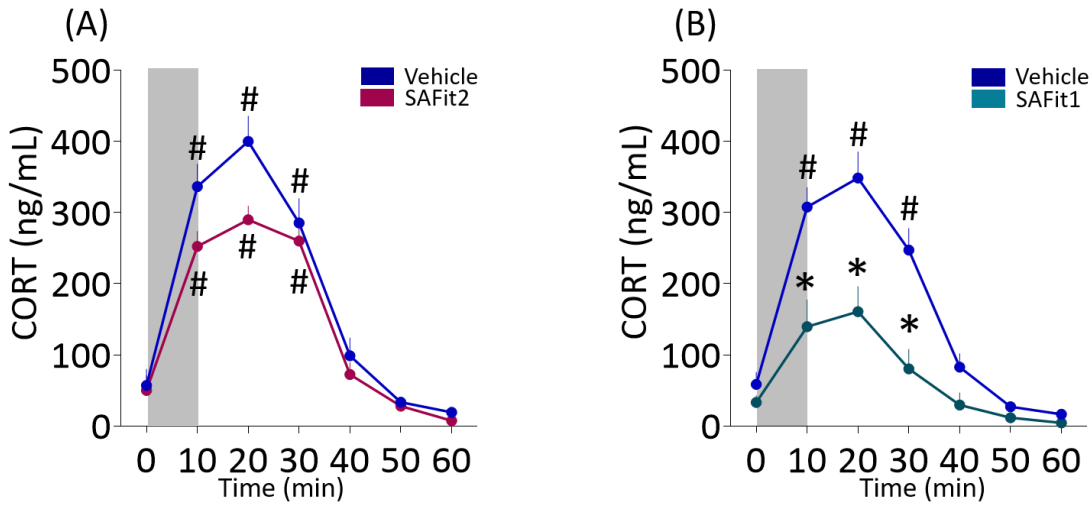

Figure 5.7. The effect of acute SAFit2 and SAFit1 treatment on stress-induced CORT secretion in female rats. *(A) Mean + SEM corticosterone levels during noise stress (96dB, 10 minutes, white noise, 07.00h) from female adult rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=7) or SAFit2 (20 mg/kg, s.c.; n=7) at 09.00h, and 17.00h on the day prior to the noise stress. (B) Mean + SEM corticosterone levels during a noise stress (96dB, 10 minutes, white noise, 07.00h) from female adult rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=3+7) or SAFit1 (20 mg/kg, s.c.; n=4) at 09.00h, 14.00h, and 19.00h on the day prior to the noise stress. Blood samples were taken every 10 minutes from a cannula implanted in the right jugular vein and CORT concentrations measured with CORT RIA. The grey bar indicates noise stress. *P<0.05; compared to rats treated with vehicle, with Tukey's post hoc test. #P<0.05; compared to respective time 0, with Tukey's post hoc test.*

5.3.4. The effect of sub-chronic SAFit2 treatment on basal CORT concentrations and ultradian rhythms in adult female rats

Six vehicle-treated rats and six SAFit2-treated rats were treated for 5 consecutive days and on the fifth day they were all subject to ABS every 10 minutes for 24 hours. The blood samples were assayed for CORT concentrations and the mean was calculated for each time point for both groups. The resultant average 24-h profiles are shown in figure 5.8 (A) along with the individual representative profile (B-E).

All the individual 24-hour profiles were analysed with PULSAR and the means for each parameter are shown in figure 5.9. Further, an unpaired t-test revealed a significant effect of treatment on mean CORT ($t(11)=2.96$, $P=0.013$), max CORT ($t(11)=3.50$, $P=0.005$), baseline CORT ($t(11)=2.68$, $P=0.021$), pulse amplitude ($t(11)=3.58$, $P=0.004$), pulse height ($t(11)=4.00$, $P=0.002$), pulse area ($t(11)=2.36$, $P=0.038$), AUC basal ($t(11)=2.11$, $P=0.048$), AUC total ($t(11)=2.31$, $P=0.046$). There was a trend of effect on the pulse length ($t(11)=1.91$, $P=0.081$) and the IPI ($t(11)=1.87$, $P=0.088$). All these parameters were decreased in the SAFit2-treated female rats. There were no significant effects on the number of pulses ($t(11)=-1.61$, $P=0.136$), or the pulse frequency ($t(11)=-1.61$, $P=0.136$).

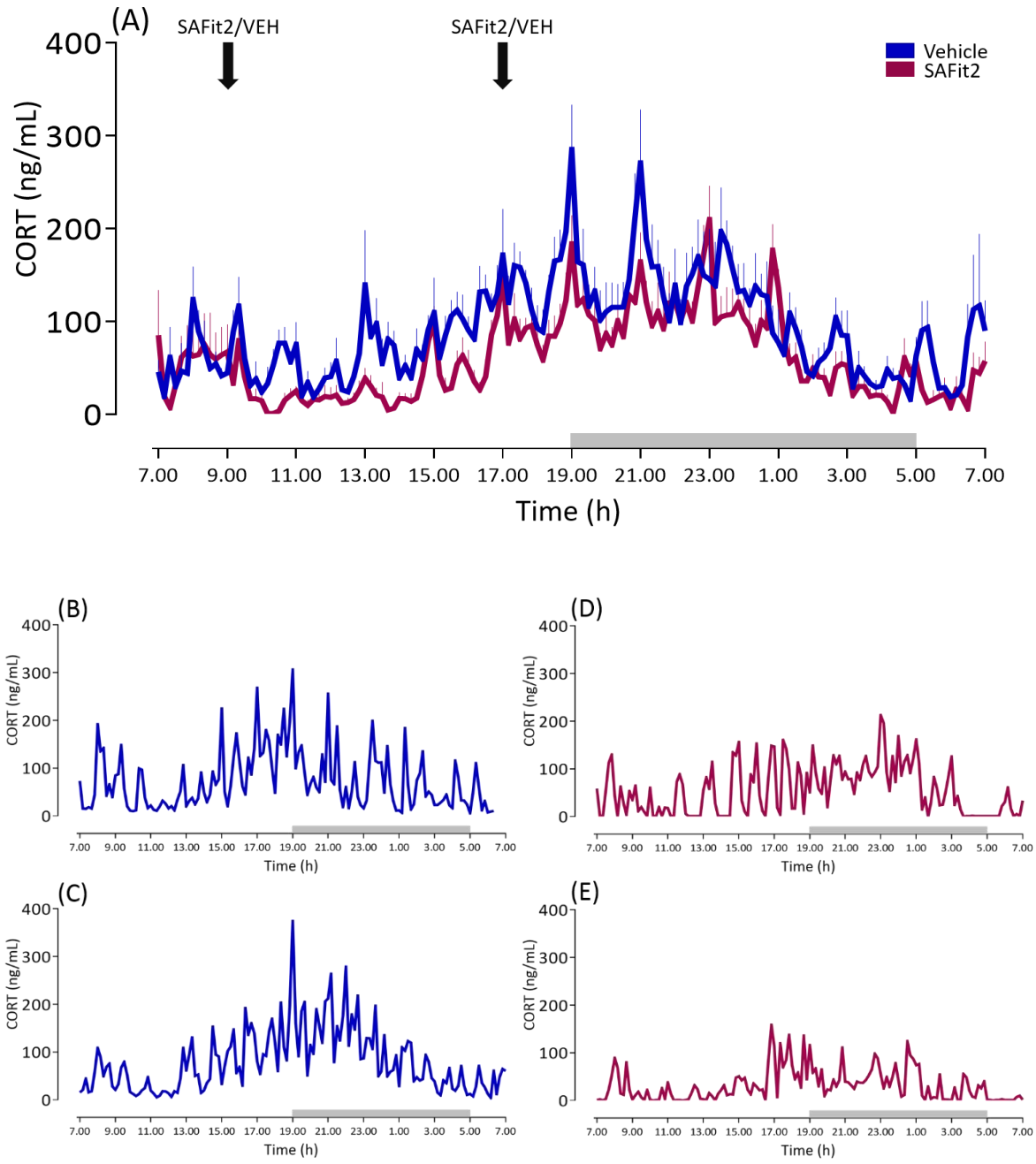

Figure 5.8. The effect of sub-chronic SAFit2 treatment on ultradian CORT secretion in female rats. *Mean + SEM (A) CORT concentrations, and representative individual profiles (B-E) from female adult rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=6) or SAFit2 (20 mg/kg, s.c.; n=7) or at 09.00h, and 17.00h for five consecutive days. Blood samples were collected via a cannula implanted in the right jugular vein every 10 min for 24-h using an automated blood sampling system on the fifth day. Plasma corticosterone was measured by radioimmunoassay (RIA). Grey bar indicates light off (19:00-05:00h).*

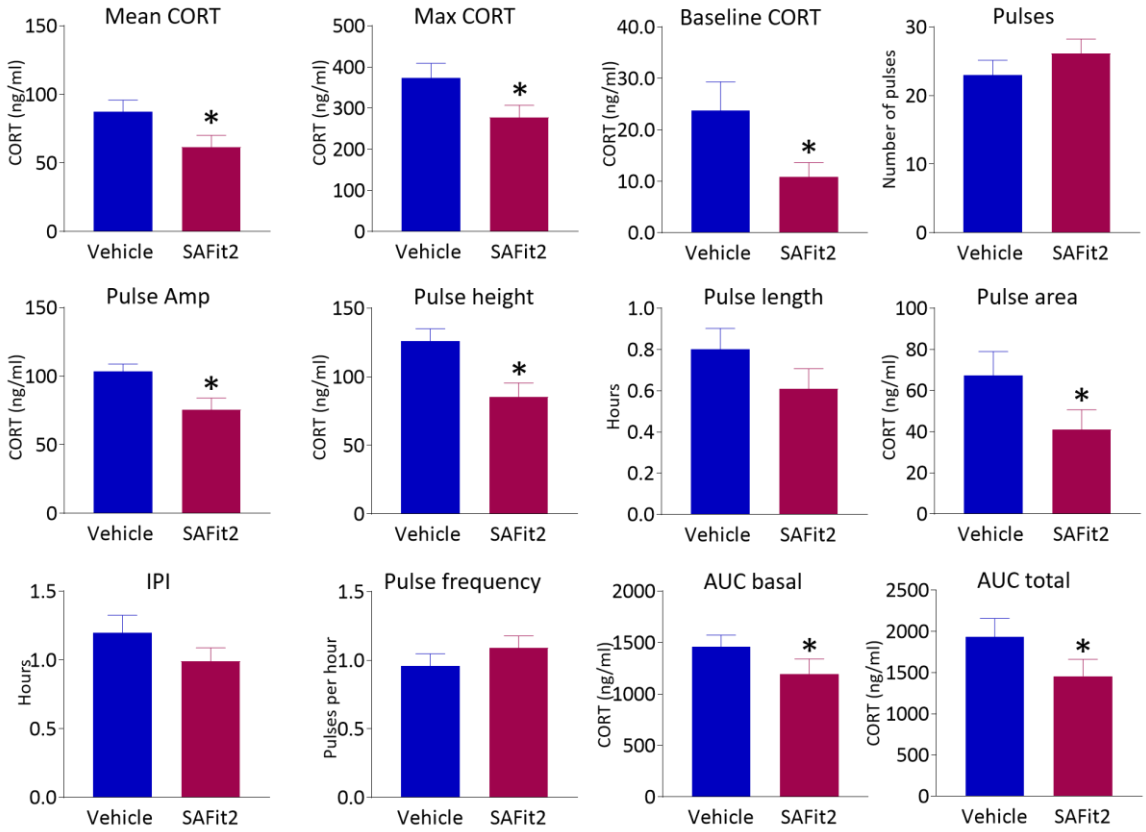

Figure 5.9. The effect of sub-chronic SAFit2 treatment on PULSAR parameters in female rats. *Mean + SEM CORT parameters calculated using PULSAR from 24-hour profiles of rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=6) or SAFit1 (20 mg/kg, s.c.; n=7) at 09.00h, and 17.00h. Amp=amplitude, IPI=inter-pulse interval, AUC= area under the curve. Amp=amplitude, IPI= inter-pulse interval, AUC= area under the curve. *P<0.05 compared to vehicle-treated rats, with unpaired t-test.*

5.3.5. The effect of sub-chronic SAFit2 treatment on post-stress CORT concentrations in adult female rats

Following sub-chronic treatment with SAFit2 in male rats, the post-noise CORT concentrations were significantly lower in the SAFit2 group. Therefore, it was of interest to investigate whether the sub-chronic treatment with SAFit2 would have the same inhibitory effect also in female rats.

Following 24-h blood sampling and treatment with SAFit2 or vehicle, the rats were subjected to noise stress and blood sampling continued for 60 minutes after the noise stress (see schematic figure 5.2).

The mean + SEM CORT concentration at each time point was calculated for each group as shown in figure 5.8. Repeated measures analysis revealed a significant effect of time ($F(3.3,26.1)= 55.3, P=<0.00001$), no significant interaction between time and treatment ($F(3.3,26.1)=0.4$, $P=0.752$), and no significant effect of treatment ($F(1,8)=0.624, P=0.448$). Further, post hoc analysis revealed a significant increase in stress-induced CORT in vehicle-treated rats at the 10 ($P<0.0001$), and 20 ($P<0.0001$) minute time points, compared to vehicle-treated rats at the 0 minute time point. A significant increase in stress-induced CORT in SAFit2-treated rats was also observed at the 10 ($P<0.0001$), 20 ($P<0.0001$), and 30 ($P=0.019$) minute time points, compared to SAFit2-treated rats at the 0 minute time point. There were no difference between the baseline CORT at the 0 minute time point, or any of the other time-points, between vehicle-treated rats and SAFit2-treated rats.

The results of this experiment indicate that repeated sub-chronic administration of SAFit2 does not affect stress-induced CORT concentrations in female rats.

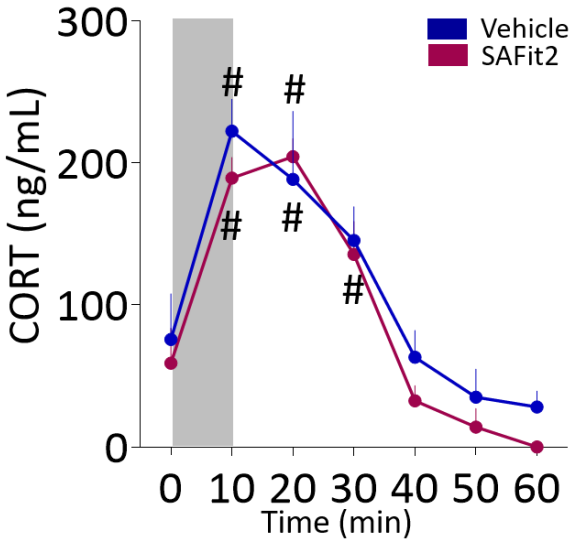

Figure 5.10. The effect of sub-chronic SAFit2 treatment on stress-induced CORT secretion in female rats. *Mean + SEM corticosterone levels during a noise stress (96dB, 10 minutes, white noise, 07.00h) from female adult rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=6) or SAFit2 (20 mg/kg, s.c.; n=6) at 09.00h, and 17.00h for five consecutive days. Blood samples were taken every 10 minutes from a cannula implanted in the right jugular vein and CORT concentrations measured with CORT RIA. The grey bar indicates noise stress. #P<0.05; compared to respective time 0, with Tukey's post hoc test.*

Numerous rodent studies have shown a sexual dimorphism in the CORT response to stress. Indeed, several studies have shown a higher response in female rats when exposed to stressors including forced running, foot shock, immunological stressors, immobilisation, alcohol administration, ether, noise stress and a novel environment (231). Comparing the data from the female sub-chronic SAFit2 experiment with the male sub-chronic SAFit2 experiment, the vehicle-treated females do not have higher post-stress CORT concentrations. That is surprising however these are different experiments on different litters, and the CORT concentrations were measured in different CORT RIAs, thus cannot be directly compared. It is, however, interesting to note the difference between the sub-chronic SAFit2 effects on the stress-response in male and female rats. Following acute SAFit2 treatment in female rats, there was a small decrease in stress-induced CORT secretion, whilst in the sub-chronically

SAFit2-treated females this was abolished and there was no difference in stress-induced CORT compared to the vehicle-treated groups. In contrast, in the acutely SAFit2-treated males, there was also a small decrease in stress-induced CORT, however, this was further lowered in the sub-chronically treated SAFit2 males. Hence, this further supports the hypothesis that SAFit2 has opposite actions centrally and peripherally in female rats, with a decrease of GC-mediated negative feedback centrally, and an increase in GC-mediated negative feedback peripherally.

5.3.6. Stress-induced gene expression in the hypothalamus and anterior pituitary gland following acute SAFit2 or SAFit1 treatment.

In both experiments, the rats were sacrificed immediately following the collection of the last blood sample, 60 minutes subsequent the noise stress. Tissues were dissected and extracted for RT-qPCR analysis for gene expression in the hypothalamus and anterior pituitary gland. As described in the sampling experiment section, due to time constraints only three vehicle-treated rats from the SAFit1 experiment were obtained and therefore the data from these rats were pooled with the data from the vehicle-treated rats from the SAFit2 experiment.

The effects of SAFit2 and SAFit1 treatment on HPA axis activity in the hypothalamus were investigated by quantifying the expression of the GR and MR, and CRH, AVP, and c-Fos. The c-Fos expression is a marker of general neuronal excitability, with an increased expression suggesting an increased neuronal firing. To evaluate whether either compound had any effect on GR transcriptional activity, the expression of several GR-inducible genes, including FKBP5, GILZ, PER1, and SGK1 was also investigated.

5.3.6.1. Hypothalamus

The relative expression of GR, MR, CRH, AVP, and c-Fos mRNA in the hypothalamus following the noise stress in the acute female SAFit2 experiment are shown in figure 5.11 A. An unpaired t-test revealed no effect of treatment for GR mRNA ($t(13)=0.53$, $P=0.600$), MR

mRNA (t(13)=0.43, P=0.672), CRH mRNA (t(13)=1.37, P=0.193), AVP mRNA (t(13)=-0.07, P=0.944), or on c-Fos mRNA (t(13)=-0.25, P=0.810).

Figure 5.11 B shows the expression of the same genes in the hypothalamus from the acute female SAFit1 experiment. A lower expression of CRH mRNA (t(12)=2.73, P=0.018) was observed in the SAFit1-treated rats compared to vehicle-treated rats. There was no effect of treatment on GR mRNA (t(12)=1.61, P=0.134), MR mRNA (t(12)=1.61, P=0.132), AVP (t(12)=-1.38, P=0.193), or on c-Fos mRNA (t(12)=1.15, P=0.270), as shown by an unpaired t-test.

The GR-inducible genes FKBP5, GILZ, PER1, and SGK1 mRNA in the hypothalamus following the noise stress in the acute female SAFit2 experiment are shown in figure 5.11 C. There was no effect of treatment on FKBP5 mRNA (t(13)=-0.95, P=0.359), GILZ mRNA (t(13)=1.03, P=0.320), PER1 mRNA (t(13)=-0.25, P=0.580), or on SGK1 mRNA (t(13)=0.16, P=0.359), as revealed by an unpaired t-test.

For the acute female SAFit1 experiment, the expression of the same GR-inducible genes in the hypothalamus following noise stress are shown in figure 5.11 D. An unpaired t-test revealed a trend for an effect of treatment on FKBP5 mRNA (t(12)=-1.86, P=0.087), with higher expression in the SAFit1-treated rats compared to vehicle-treated rats. There was no effect of treatment on GILZ mRNA (t(12)=0.50, P=0.626), PER1 mRNA (t(12)=0.72, P=0.486), or on SGK1 mRNA (t(12)=1.65, P=0.125).

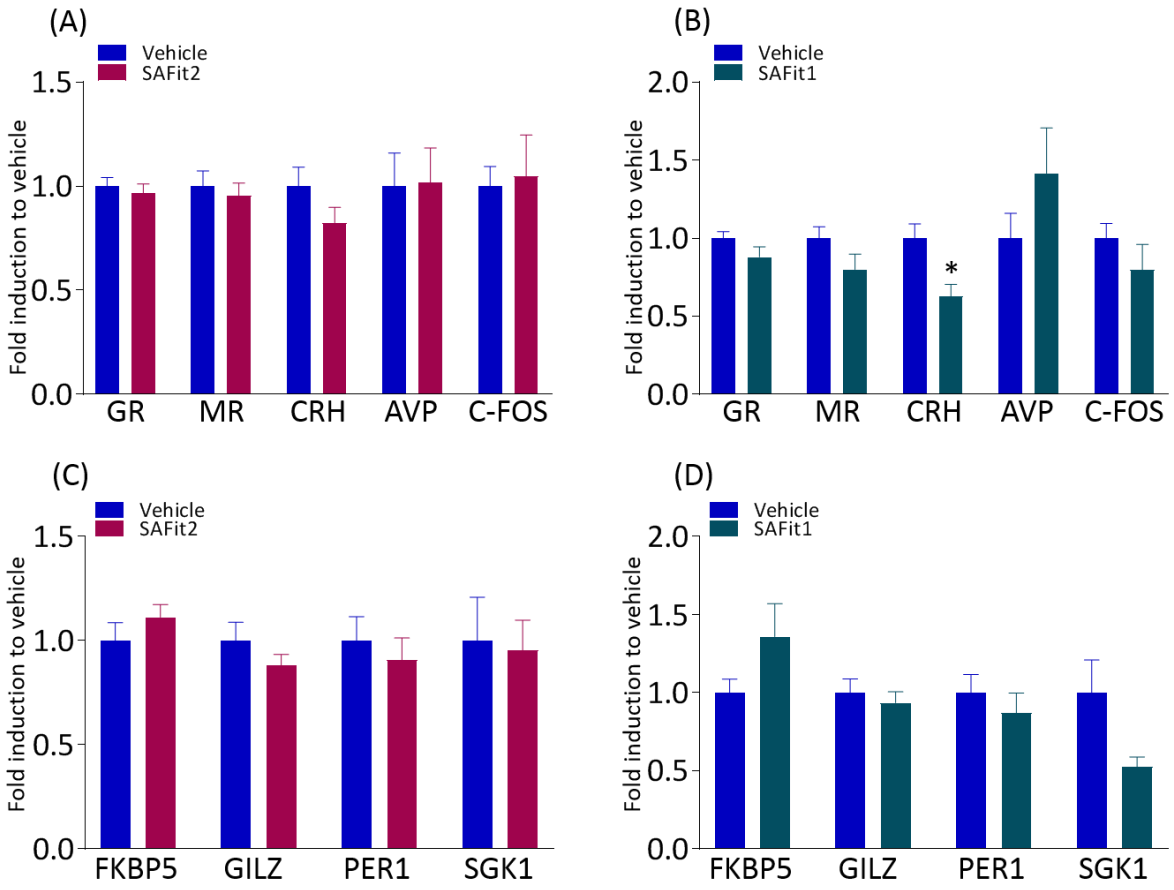

Figure 5.11. The effect of acute SAFit2 and SAFit1 treatment on stress-induced gene expression in the hypothalamus of female rats. *Mean + SEM of GR, MR, CRH, AVP, and c-Fos mRNA in the hypothalamus of female rats treated acutely with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=7) or SAFit2 (20 mg/kg, s.c.; n=7) (A) and vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=3+7) or SAFit1 (20 mg/kg, s.c.; n=4)(B). Mean + SEM of FKBP5, GILZ, PER1, and SGK1 mRNA in the hypothalamus of female rats treated acutely with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=7) or SAFit2 (20 mg/kg, s.c.; n=7) (C) and vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=3+7) or SAFit1 (20 mg/kg, s.c.; n=4)(D). *P<0.05 compared to vehicle, with unpaired t-test.*

5.3.6.2. Anterior pituitary gland

In the anterior pituitary gland, the expression of GR, MR, CRH, AVP, and c-Fos mRNA following noise stress in the acute female SAFit2 experiment is shown in figure 5.12 A.

Unpaired t-tests revealed a significant effect of treatment on POMC mRNA ($t(13)=2.49$, $P=0.027$), with lower expression in the SAFit2 group. There was no effect of treatment on GR mRNA ($t(13)=0.06$, $P=0.950$), MR mRNA ($t(13)=0.27$, $P=0.786$), or on c-Fos mRNA ($t(13)=-1.75$, $P=0.103$).

The relative expression of GR, MR, CRH, AVP, and c-Fos mRNA in the anterior pituitary gland following noise stress in the acute female SAFit1 experiment are shown in figure 5.12 B. There was no effect of treatment on GR mRNA ($t(12)=-0.75$, $P=0.467$), MR ($t(12)=-1.32$, $P=0.211$), POMC ($t(12)=-0.39$, $P=0.721$), or on c-Fos mRNA ($t(12)=-1.24$, $P=0.239$), as revealed by unpaired t-tests.

Figure 5.12 C shows the expression of FKBP5, GILZ, PER1, and SGK1 mRNA in the anterior pituitary gland following noise stress in the acute female SAFit2 experiment. Unpaired t-tests revealed no effect of treatment on either of the genes: FKBP5 mRNA ($t(13)=-0.07$, $P=0.944$), GILZ mRNA ($t(13)=0.58$, $P=0.572$), PER1 mRNA ($t(13)=0.52$, $P=0.359$), or on SGK1 mRNA ($t(13)=-1.05$, $P=0.313$).

Further, for the acute SAFit1-treated female rats the relative expression of FKBP5, GILZ, PER1, and SGK1 mRNA in the anterior pituitary gland following the noise stress are shown in figure 5.12 D. An unpaired t-test revealed a significant effect of treatment on FKBP5 mRNA ($t(12)=-2.96$, $P=0.012$), with higher expression in the SAFit1-treated rats compared to vehicle-treated rats. There was a trend for an effect of treatment on SGK1 mRNA ($t(12)=1.95$, $P=0.074$), with decreased expression in the SAFit1-treated rats compared to vehicle-treated rats. There was no effect of treatment on GILZ mRNA ($t(12)=-1.65$, $P=0.125$), or on PER1 mRNA ($t(12)=-1.49$, $P=0.161$).

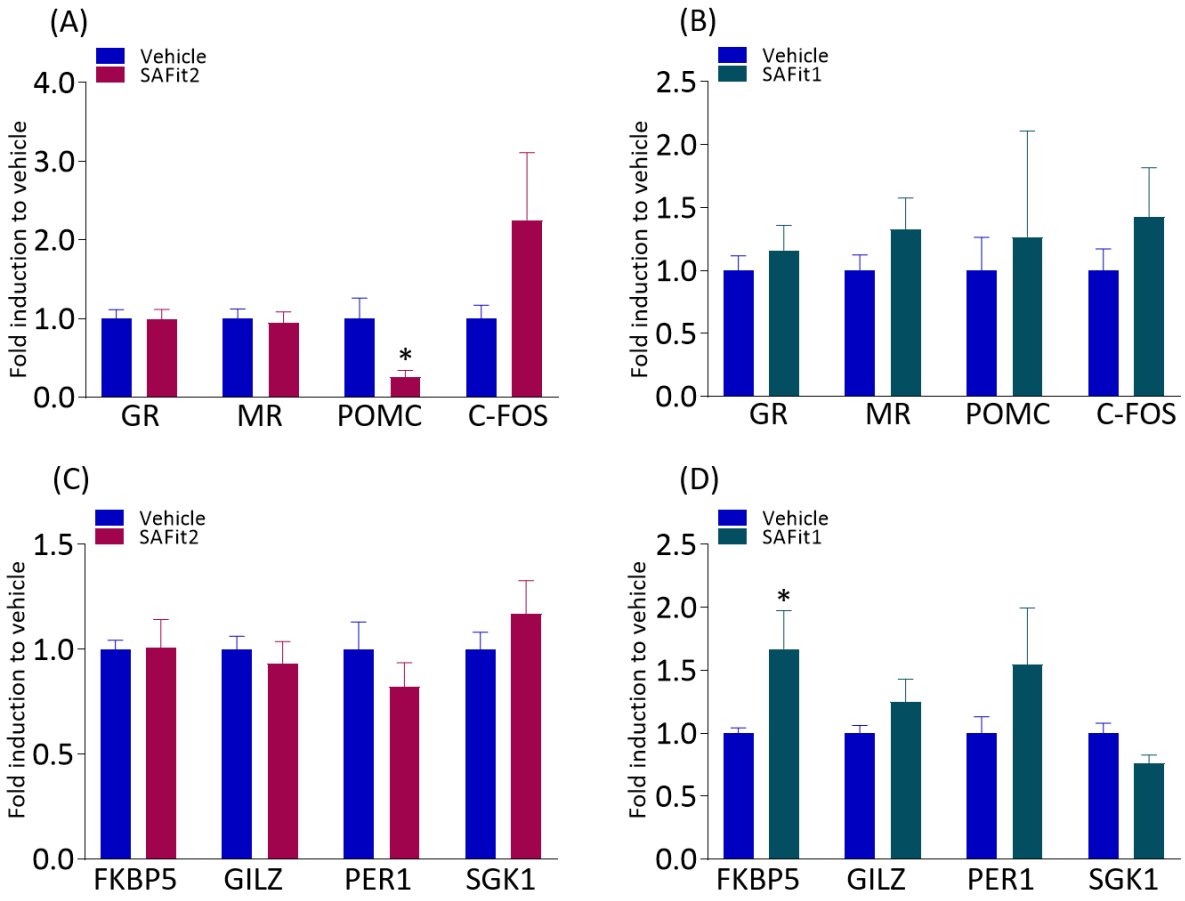

Figure 5.12. The effect of acute SAFit2 and SAFit1 treatment on stress-induced gene expression in the anterior pituitary gland of female rats. *Mean + SEM of GR, MR, CRH, AVP, and c-Fos mRNA in the anterior pituitary gland of female rats treated acutely with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=7) or SAFit2 (20 mg/kg, s.c.; n=7) (A) and vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=3+7) or SAFit1 (20 mg/kg, s.c.; n=4)(B). Mean + SEM of FKBP5, GILZ, PER1, and SGK1 mRNA in the anterior pituitary gland of female rats treated acutely with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=7) or SAFit2 (20 mg/kg, s.c.; n=7) (C) and vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=3+7) or SAFit1 (20 mg/kg, s.c.; n=4)(D). *P<0.05 compared to vehicle, with unpaired t-test.*

These data indicate an effect of the acute SAFit2 treatment on POMC mRNA, with lower expression in the anterior pituitary gland in the SAFit2 group, suggesting the SAFit2

might increase negative feedback at the level of the pituitary gland in female rats by reducing POMC mRNA expression and decreased ACTH synthesis and secretion.

Furthermore, these results indicate that there is a pituitary gland-specific induction of FKBP5 in the acute SAFit1 group following noise stress. This indicates either increased transcriptional GR activity or compensation for reduced FKBP51 activity by enhancing FKBP5 mRNA production. Nevertheless, it is plausible to speculate that SAFit1 affects negative feedback in the pituitary gland to reduce CORT concentrations. There was no effect on FKBP5 mRNA in the anterior pituitary gland following acute SAFit2 treatment, suggesting that the two SAFit compounds have different effects on the regulation of this gene. This is consistent with the lower basal and stress-induced CORT concentration observed in the SAFit1 group but not in the SAFit2 group.

5.3.7. Stress-induced gene expression in the hypothalamus and anterior pituitary gland following sub-chronic SAFit2 treatment

To compare the effect of acute SAFit2 treatment with sub-chronic SAFit2 treatment on HPA axis activity in the hypothalamus and anterior pituitary gland in females, the expression of GR, MR, CRH, AVP, and c-Fos was quantified. The c-Fos expression is a marker of general neuronal excitability, with an increased expression indicating increased neuronal firing (378). To evaluate whether either compound had any effect of GR transcriptional activity, expression of some GR-inducible genes, including FKBP5, GILZ, PER1, and SGK1 was also investigated.

Figure 5.13 A represents the expression of GR, MR, CRH, AVP, and c-Fos mRNA in the hypothalamus following the noise stress in the sub-chronic female SAFit2 experiment. An unpaired t-test revealed no effect of treatment on GR mRNA ($t(14)=-0.81$, $P=0.434$), MR mRNA ($t(14)=-0.29$, $P=0.775$), CRH mRNA ($t(14)=1.04$, $P=0.315$), AVP mRNA ($t(14)=0.15$, $P=0.882$), or on c-Fos mRNA ($t(14)=0.87$, $P=0.487$).

Furthermore, the relative expression of FKBP5, GILZ, PER1, and SGK1 mRNA in the hypothalamus for the same experiment are shown in figure 5.13 B. There was no effect of treatment on FKBP5 mRNA ($t(14)=1.02$, $P=0.322$), GILZ mRNA ($t(14)=0.575$, $P=0.574$), PER1

mRNA (t(14)=0.186, P=0.855), or on SGK1 mRNA (t(14)=0.37, P=0.717) as revealed by unpaired t-test.

For the anterior pituitary gland, the relative expression of GR, MR, CRH, AVP, and c-Fos mRNA in the hypothalamus following noise stress in the sub-chronic female SAFit2 experiment are shown in figure 5.13 C. An unpaired t-test revealed no effect of treatment on GR mRNA (t(14)=0.47, P=0.640), MR mRNA (t(14)=0.34, P=0.735), POMC mRNA (t(14)=-1.55, P=0.141), or on c-Fos mRNA (t(14)=0.99, P=0.337).

Lastly, figure 5.13 D shows the expression of the GR-inducible genes: FKBP5, GILZ, PER1, and SGK1, in the anterior pituitary gland for the same experiment. There was a significantly higher expression of PER1 (t(14)=-2.26, P=0.040) in the SAFit2-treated rat compared to vehicle-treated rats. There was no effect of treatment on FKBP5 mRNA (t(14)=0.17, P=0.865), GILZ mRNA (t(14)=-0.17, P=0.862), or on SGK1 mRNA (t(14)=0.85, P=0.407).

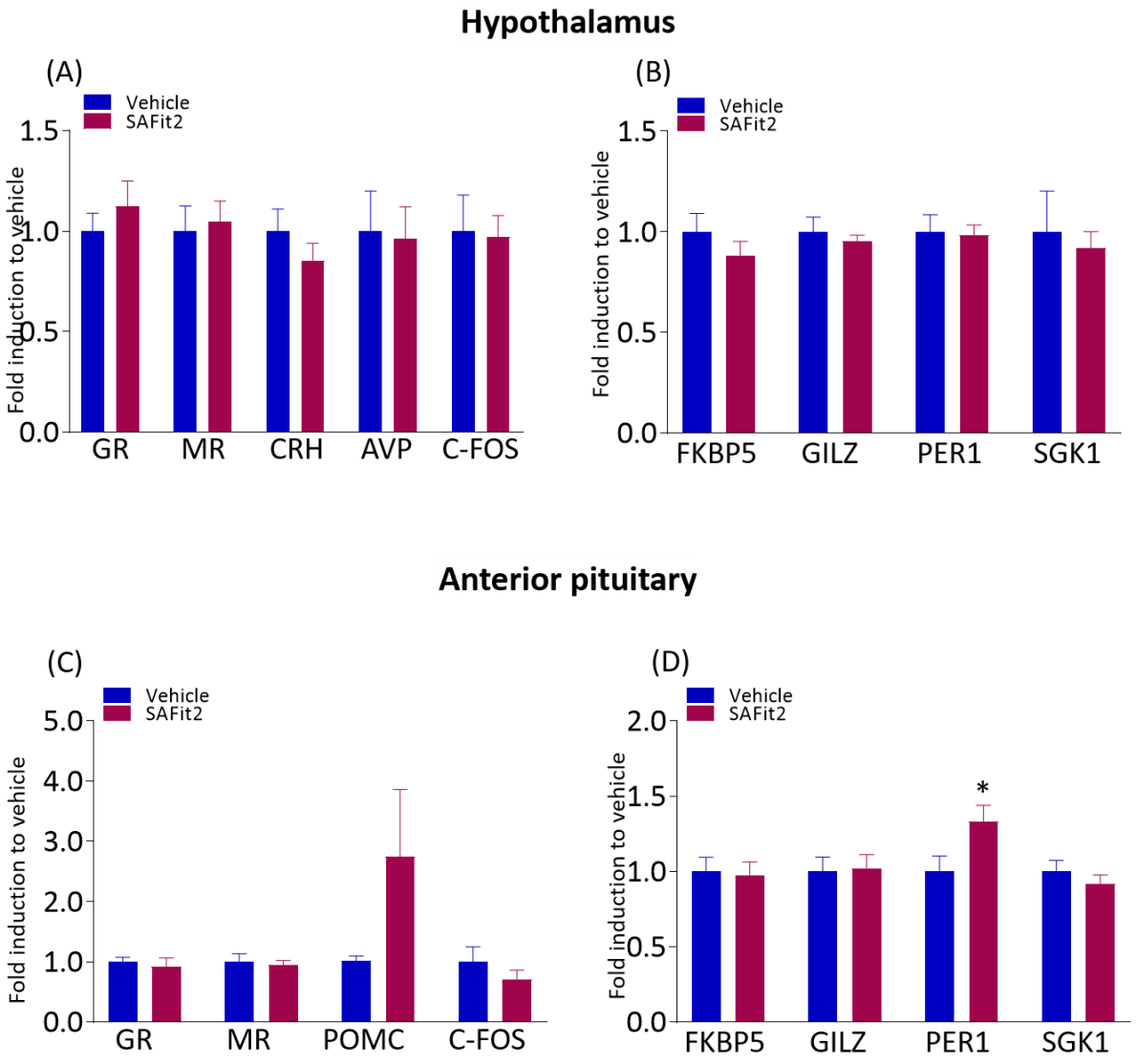

Figure 5.13. The effect of sub-chronic SAFit2 treatment on stress-induced gene expression in the hypothalamus and anterior pituitary gland of female rats. *Mean + SEM of GR, MR, CRH, AVP, and c-Fos mRNA in the hypothalamus (A) and anterior pituitary gland (C) of female rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=6) or SAFit2 (20 mg/kg, s.c.; n=6) for 5 consecutive days. Mean + SEM of FKBP5, GILZ, PER1, and SGK1 mRNA in the hypothalamus (B) and anterior pituitary gland (D) of female rats treated with vehicle (VEH: 4% Ethanol; 5% PEG300; 5% Tween 80 in saline; 2 ml/kg; s.c.; n=6) or SAFit2 (20 mg/kg, s.c.; n=6) for 5 consecutive days. *P<0.05 compared to vehicle, with unpaired t-test.*

In summary, there are no changes in gene expression at 60 minutes post noise stress in the hypothalamus following sub-chronic SAFit2 treatment. In contrast, an increase of PER1 in the anterior pituitary gland was observed in the SAFit2-treated rats compared to the vehicle-treated rats. This suggests an increase in GR transcriptional activity only in the pituitary gland following stress in sub-chronic SAFit2-treated females.

5.3.8. Sexual dimorphism in the expression of GR, MR, and FKBP51

For this study, the aim was to investigate whether the sexual dimorphism in the effect of SAFit1 and Safit2 on HPA axis activity may be due to differential expression of the GR, MR or FKBP51 in male and female rats. The expression of these genes was quantified in the brains and anterior pituitary gland of adult, untreated intact male and female rats, sacrificed either in the morning or the afternoon. This experimental design allowed for the investigation of not only the sex differences in gene expression but also the circadian expression of GR, MR and FKBP5, and if that expression differs in males and females. In addition to the anterior pituitary gland, other brain areas were investigated, including the hippocampus, hypothalamus, and prefrontal cortex, as these are all known to project to the PVN and to regulate HPA axis activity (21).

Hippocampus

The expression of hippocampal FKBP5, GR, and MR is shown in figure 5.14. Two-way ANOVA for FKBP5 gene expression in the hippocampus showed that there was an overall effect of time ($F(1,24)=6.311$, $P=0.021$), but no effect of sex ($F(1,24)=0.065$, $P=0.801$), nor any interaction ($F(1,24)=0.824$, $P=0.375$). Further post hoc analysis revealed no significant differences between the groups. From this it can be deduced that there was a difference in the expression of FKBP5 in the hippocampus in the morning and evening, however, the expression level was similar in both sexes.

With regard to hippocampal GR expression there was no significant effect of time ($F(1,24)=2.738$, $P=0.114$), and no significant effect of sex ($F(1,24)=0.614$, $P=0.442$), however there was a significant interaction ($F(1,24)=5.777$, $P=0.026$). Further post hoc analysis revealed a significant difference in GR expression between AM and PM in the female rats ($P=0.043$), with lower levels in the PM, whereas no such difference was found in the males.

This suggests that in the hippocampus there is a circadian rhythm of GR expression with lower levels in the evening in females but not in males. The hippocampus generally has a net inhibitory input to the HPA axis, and the deletion of hippocampal GR in the mouse leads to increased CORT levels in the morning (390). Thus in females perhaps lower GR expression in the evening, compared to the morning, contributes to a bigger difference in circadian CORT fluctuations compared to male rats.

There was no difference in MR expression in the hippocampus in either of the groups as demonstrated by no effect of time ($F(1,24)=2.781$, $P=0.111$), sex ($F(1,24)=0.170$, $P=0.684$) and no interaction ($F(1,24)=0.002$, $P=0.969$), suggesting that MR expression in the hippocampus are similarly expressed in both sexes. Previously it has been found in male rats that MR mRNA expression in all hippocampus subfields has a circadian pattern with higher levels in the morning, a trough at around 15.00 and then the expression increases to levels similar to the morning (360). This is consistent with the findings in the present study, as the PM time point is around 17.00h and, therefore, the MR mRNA levels may have risen again following the trough.

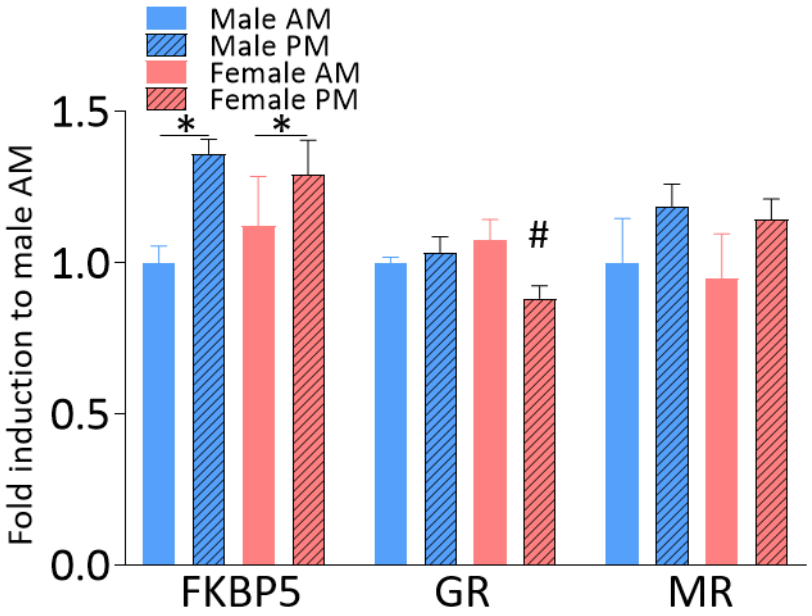

Figure 5.14. Expression of FKBP5, GR, and MR in the hippocampus of male and female rats. *Mean + SEM of FKBP5, GR, and MR mRNA in the hippocampus, in male AM (n=6), male PM (n=6), female AM (n=6), and female PM (n=6). Animals were euthanized in the AM (08.30h to 09.30h) or the PM (16.30h to 17.30h) and tissues snap-frozen on dry ice. #P<0.05 compared to female AM. *P<0.05 effect of time.*

Hypothalamus

Given the role of PVN CRH and AVP in the regulation of the HPA axis the expression of FKBP5, GR, and MR in the hypothalamus was investigated (figure 5.15). Two-way ANOVA for FKBP5 expression in the hypothalamus revealed an overall effect of time ($F(1,24)=8.588$, $P=0.008$), however, there was no effect of sex ($F(1,24)=0.593$, $P=0.450$), and no significant interactions ($F(1,24)=1.531$, $P=0.230$). Post hoc analysis revealed significantly higher expression in the female PM compared to female AM ($P=0.037$), but this difference was not observed in the males. This indicates that in females there may be a circadian difference in the FKBP5 mRNA expression in the hypothalamus, with higher expression in the evening, while in the males the expression may be flattened. Higher levels of FKBP5 could potentially contribute to the higher CORT concentrations seen in female rats, by decreasing GR activity and hence reducing negative feedback.

Further, there was no effect of time on hypothalamic GR expression ($F(1,24)=0.686$, $P=0.417$), no effect of sex ($F(1,24)=1.047$, $P=0.318$), and no interaction ($F(1,24)=1.199$,

P=0.287). Similarly, for hypothalamic MR expression there was no effect of time (F(1,24)=0.106, P=0.748), no effect of sex (F(1,24)=0.461, P=0.505), and no interaction (F(1,24)=1.659, P=0.212). These results suggest that the underlying sex differences in the HPA axis in male and female rats are not due to differential GR and MR expression in the hypothalamus.

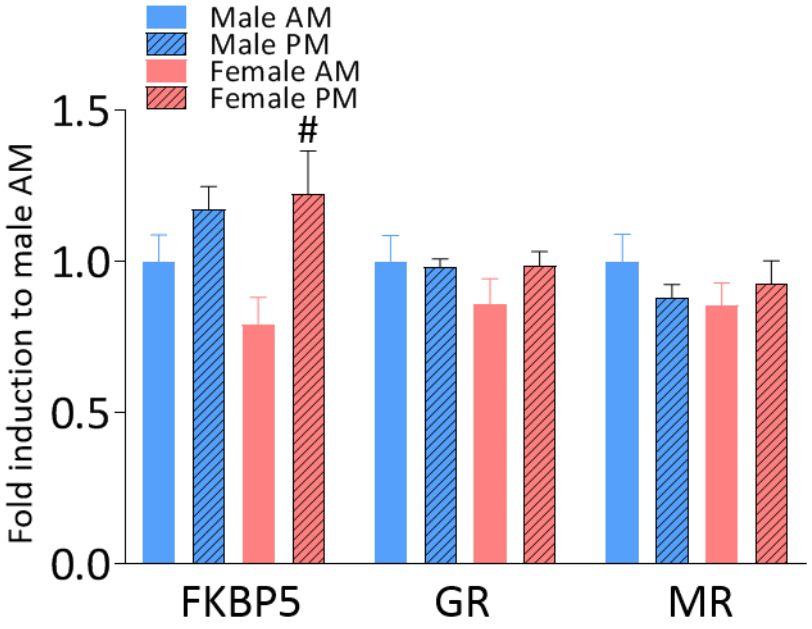

Figure 5.15. Expression of FKBP5, GR, and MR in the hypothalamus of male and female rats. *Mean + SEM of FKBP5, GR, and MR mRNA in the hypothalamus, in male AM (n=6), male PM (n=6), female AM (n=6), and female PM (n=6). Animals were euthanized in the AM (08.30h to 09.30h) or the PM (16.30h to 17.30h) and tissues snap-frozen on dry ice. #P<0.05 compared to female AM.*

Anterior pituitary gland

As suggested by the data reported here, the effects of SAFit1 and SAFit2 are presumably mainly due to an effect of the compounds on the anterior pituitary gland, supporting the general idea that the GR activity in the pituitary gland is important for regulation of basal CORT concentrations. It was therefore of interest to elucidate whether the expression of FKBP5, MR, and GR in the anterior pituitary gland was different in female rats compared to male rats. The expression of anterior pituitary gland FKBP5, GR, and MR mRNA

is shown in figure 5.16. Two-way ANOVA for anterior pituitary gland FKBP5 expression revealed no effect of time ($F(1,24)=2.514$, $P=0.129$), however there was a significant effect of sex ($F(1,24)=4.688$, $P=0.043$), but not an interaction ($F(1,24)=0.273$, $P=0.607$). The post hoc analysis did reveal a trend towards an effect of lower FKBP5 in the female rats in the PM group, compared to the male rats in the PM group ($P=0.072$). Similarly, results for anterior pituitary gland GR mRNA expression showed no effect of time ($F(1,24)=1.553$, $P=0.417$), an overall effect of sex ($F(1,24)=4.443$, $P=0.048$), and no interaction ($F(1,24)=2.008$, $P=0.172$). The post hoc test failed to reveal any significant difference for the specific groups, however, there was a trend for lower GR expression in females PM compared to males PM ($P=0.070$). For anterior pituitary gland MR mRNA expression there was no effect of time ($F(1,24)=0.916$, $P=0.350$), however an overall significant effect of sex ($F(1,24)=7.154$, $P=0.015$), and no interaction ($F(1,24)=0.750$, $P=0.397$). The post hoc analysis revealed significantly lower MR mRNA in the female rats in the PM group compared to male rats in the PM group ($P=0.021$).

It is somewhat surprising that the expression of FKBP5 is lower overall in the females compared to males at the time points investigated, as lower FKBP5 would be expected to be accompanied by increased GR translocation and activity and increased CORT-mediated negative feedback. However, the anterior pituitary gland GR mRNA expression is also lower in the females compared to the males which would potentially cause lower GR activity. It is plausible that the lower FKBP5 expression is to compensate for the lower GR expression. Interestingly it has been found that ovariectomized female rats have higher anterior pituitary gland GR and MR mRNA expression compared to ovariectomized females given oestrogen replacement (391). This suggests that oestrogen in females is involved in the regulation of GR and MR mRNA expression in the anterior pituitary gland. This is also consistent with the data showing higher levels of anterior pituitary gland GR and MR mRNA in males with no oestrogen, similar to the ovariectomized females. This is also consistent with several studies demonstrating that oestrogen increases HPA axis activity and testosterone has an inhibitory effect on the HPA axis (reviewed in (231)) and ovariectomised female rats have basal CORT concentrations similar to sham males, while castrated males display basal CORT levels similar to sham females (240).

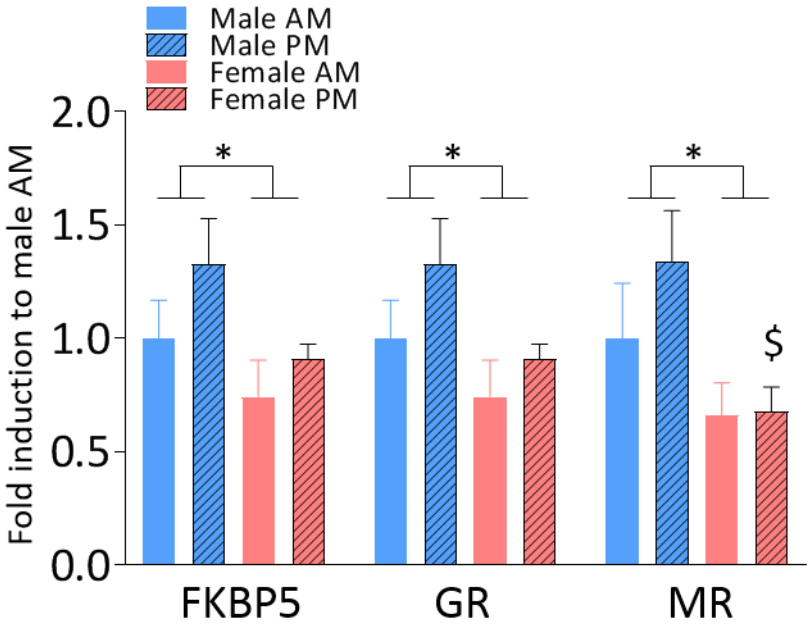

Figure 5.16. Expression of FKBP5, GR, and MR in the anterior pituitary gland of male and female rats. *Mean + SEM of FKBP5, GR, and MR mRNA in the anterior pituitary gland, in male AM (n=6), male PM (n=6), female AM (n=6), and female PM (n=6). Animals were euthanized in the AM (08.30h to 09.30h) or the PM (16.30h to 17.30h) and tissues snap-frozen on dry ice. *P<0.05 effect of sex. $P<0.05, compared to males PM.*

Prefrontal cortex

The prefrontal cortex and more specifically the medial prefrontal cortex, which is the limbic region of the prefrontal cortex, is activated following a stressor as demonstrated by enhanced c-Fos and glucose utilisation (50, 51). Lesion of the medial prefrontal cortex in male rats does not affect the basal AM or PM CORT or ACTH concentrations although the CORT and ACTH concentrations following restraint stress are significantly elevated (54, 55). These studies indicate a specific effect of the MpFC in the regulation of the stress response but not basal HPA axis activity. These effects are at least partly mediated by GR, as knockdown of the GR in either the infralimbic region or the prelimbic region of the MpFC causes hyperresponsiveness to stressful stimuli (392). Further, MR is also expressed in the prefrontal cortex and is downregulated in patients with bipolar disorder (393), implicating pre-frontocortical MR in influencing HPA axis activity. Given the role of both prefrontocortical GR and MR in HPA axis activity, the expression of FKBP51, GR, and MR mRNA were quantified

(figure 5.17). Two-way ANOVA showed no effect of time (F(1,24)=0.564, P=0.461) or of sex (F(1,24)=1.023, P=0.324) and no interaction (F(1,24)=0.005, P=0.944). These data suggest that sex differences in the stress response are not likely to be due to differences in prefrontal cortex expression of the GR or MR, or FKBP51 at the time points investigated. However, it cannot be ruled out that there is a difference in FKBP5, GR and MR expression within specific regions of the prefrontal cortex or at different time points.

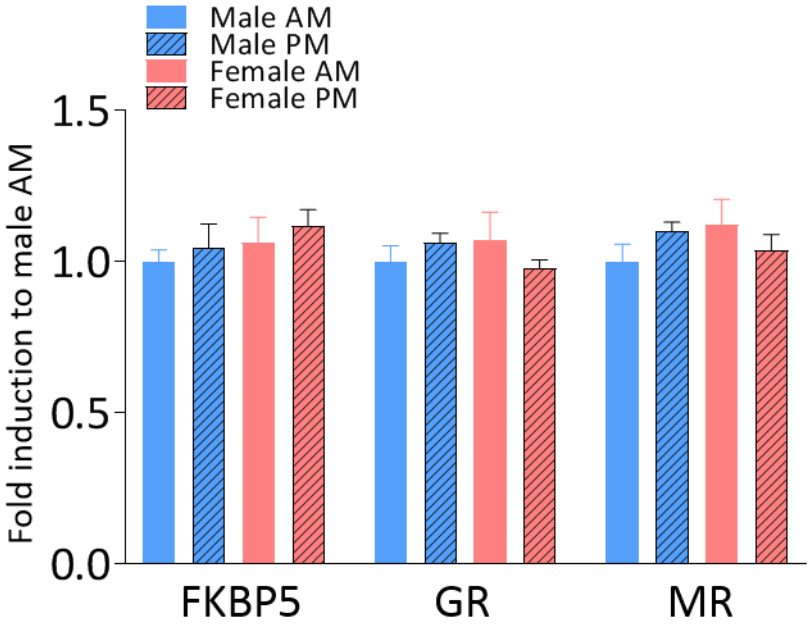

Figure 5.17. Expression of FKBP5, GR, and MR in the prefrontal cortex of male and female rats. *Mean + SEM of FKBP5, GR, and MR mRNA in the prefrontal cortex, in male AM (n=6), male PM (n=6), female AM (n=6), and female PM (n=6). Animals were euthanized in the AM (08.30h to 09.30h) or the PM (16.30h to 17.30h) and tissues snap-frozen on dry ice.*

In summary, data from this experiment highlights a sex difference in expression of GR, MR, and FKBP5 in the anterior pituitary gland, with overall lower expression of all three genes in females. Lower GR in the anterior pituitary gland might cause reduced negative feedback and may perhaps contribute to overall higher CORT concentrations in female rats. Although the expression of FKBP5 is also lower, this could counteract the lower expression of GR, however, it could also render FKBP51-inhibition less effective as there is less FKBP51 available to begin with. Interestingly one study showed a decrease in GR mRNA expression in the PVN

in castrated males compared to sham males. In contrast, an increase in GR mRNA expression was observed in ovariectomized females compared to sham females (239, 240).

5.4. Discussion

In this chapter, data has been presented demonstrating a strong effect of acute SAFit1 treatment in female rats on HPA axis activity, with lower basal and stress-induced CORT concentrations.

As already mentioned, numerous studies indicate sexual dimorphism in HPA axis activity, both in basal and in stress-induced CORT secretion. There is inconclusive evidence indicating whether this dimorphism is central and/or peripheral and if it involves GR, MR, and FKBP51. One study measured basal CORT concentrations during a 24-h period in sham males, castrated males, sham females, and castrated females, and found that castrated males showed similar CORT profiles to sham females and vice versa (239). Further, these effects were abolished by administering testosterone or oestrogen (240). Furthermore, the same effects were seen on the post-stress CORT concentrations following noise stress and LPS injections (immune stress). This suggests that sex steroids directly affect the HPA axis activity and that these effects regulate both basal and stress-induced CORT secretion (239, 240).

As mentioned in the general introduction, female 51KO mice have significantly lower concentrations of both basal and post-stress CORT (343), suggesting that a decrease in, or a complete knock out of FKBP51 alters HPA axis activity in female mice. In the present study, there was no overall difference in hypothalamic FKBP5 or GR mRNA expression in females and males, although the females had a stronger circadian difference in hypothalamic FKBP5 expression. Further, the acute SAFit1 and SAFit2 studies in male rats showed an effect of SAFit2 in the brain and anterior pituitary gland and only in the anterior pituitary gland for SAFit1. Given the lower expression of both FKBP51 and GR in females in the anterior pituitary gland, it is plausible to expect a reduced effect of the SAFit2 in females as there is lower anterior pituitary gland FKBP51 expression already and there is lower expression of anterior pituitary gland GR to be activated and induce negative feedback. Acute administration of SAFit1 in male rats did not alter either basal or stress-induced CORT concentrations. Although in males, acute SAFit1 or SAFit2 treatment on the day before CRH injection caused a repressed

ACTH and CORT response, suggesting that both SAFit2 and SAFit1 have an effect on the pituitary gland.

In chapter 3 the effects of both SAFit2 and SAFit1 on CORT secretion following an ACTH challenge in male rats were discussed. The results indicated that neither SAFit compounds have a direct effect on ACTH-induced CORT secretion from the adrenal glands. Unfortunately, due to time constraints, the same experiment could not be performed in female rats, although that would potentially have produced interesting findings. It is therefore not known whether SAFit2 and SAFit1 can affect the adrenal glands directly in the female rat. In addition, the brain permeability studies were performed in male mice, and hence SAFit1 may have a different brain permeability in rats and especially female rats.

In addition to lipid solubility, numerous other factors dictate the brain permeability of a molecule, including charge, tertiary structure, and protein binding (394). However, the main factor determining brain permeability is size, and it has been described that the cut-off point for molecules is around 400-600 kDa, although it has been proposed that few molecules over 500 kDa reach the brain by simple diffusion (395). The molecular weight of SAFit2 and SAFit1 is 803 kDa and 747 kDa, respectively (349).

Uptake transporters function to transport essential nutrients to the brain such as glucose, vitamin B6 and vitamin B12, and other substances, for example, L-DOPA and caffeine, are transported into the brain by a saturable transport system (396). The rate of diffusion of a saturable transport system can be up to 10 times higher than transmembrane diffusion (397).

On the other hand, the P-glycoprotein belongs to the family of ABC transporters and is a blood-to-brain efflux pump which can limit the amount of drug available in the brain (398). The P-glycoprotein and other ABC transporters are a major problem in the development of central nervous system drugs (399).

It is currently not known how SAFit2 enters and remains in the brain and why SAFit1 does not. SAFit2, due to its size, presumably enters the brain by active transport. Although peptides and proteins over 600kDa have been known to cross the BBB by transmembrane diffusion. For example, the cytokine-induced neutrophil chemoattractant-1 (CINC-1) with a

molecular weight of 7800kDa is the largest substance known to have crossed the BBB (400) hence it is indeed plausible that SAFit2 does not require active transport across the BBB.

In addition, SAFit2 is also likely to be actively transported out of the brain with differences between males and females. Furthermore, the plasma to brain ratio studies were performed in male mice (347) and it is not known how these parameters compare in rats. However, experiments to elucidate this are currently being planned. The current hypothesis is that SAFit1 has less membrane permeability and increased brain efflux.

The results from the acute SAFit2 ABS experiment in females indicate no effect on basal CORT secretion. SAFit2 and SAFit1 both act peripherally and only differ in BBB permeability and half-life. Hence, as SAFit1 decreases basal CORT by actions in the periphery, the central FKBP51-inhibition elicited by SAFit2 must have an opposing effect on the peripheral FKBP51-inhibition. Thus, it is plausible that central FKBP51-inhibition in female rats increases CRH release from the PVN. SAFit2 administration would then cause increased CRH secretion to the pituitary gland from central FKBP51 inhibition but decreased ACTH and CORT secretion. This would counteract the increase in CRH and result in unchanged CORT secretion, as seen in the female SAFit2 ABS study.

A study using global FKBP51 knock-out (51KO) female mice measured basal CORT concentrations and revealed lower CORT in the 51KO female (343). However, besides any species-related difference, these data are representative of only one time point of basal CORT and are from mice, which differ from the rat in various aspects of physiology. To my knowledge, there are no other findings of differential activity of FKBP51 in male and female rats and it is an area that would be interesting to investigate further.

The data in this chapter are in contrast to the results from the same experiment performed in males, where there was no difference between SAFit1- and vehicle-treated rats. In the females, however, there was no effect of acute SAFit2 on basal CORT secretion and only a small decrease in stress-induced CORT. Furthermore, the sub-chronic administration of SAFit2 caused a decreased in basal CORT but had no effect on stress-induced CORT. In addition, females treated acutely with SAFit1 had increased expression of FKBP5 mRNA following exposure to noise-stress, compared to vehicle-treated rats, while there was no effect on acutely treated SAFit2 females. In contrast, an increase in PER1 mRNA expression

following sub-chronic SAFit2 was seen in the anterior pituitary gland of the female rats. These data suggest that in females, central inhibition of FKBP51 may counteract the effect of peripheral FKBP51-inhibition. This would explain the lower CORT secretion following the acute administration of SAFit1 but not of SAFit2. However, sub-chronic SAFit2 decreased basal CORT concentrations significantly. Perhaps a prolonged treatment with SAFit2 would cause the peripheral effects to be stronger than the central effects. This is also consistent with an increase in the GR-inducible PER1 mRNA expression in SAFit2-treated animals following sub-chronic treatment.

With regards to the effect of SAFit2 and SAFit1 on the stress-induced CORT in these three different experiments, further investigation will help unravel the mechanisms underlying the role of FKBP51 and GR in the regulation of stress-induced negative feedback. However, the data from the experiments described in this chapter indicate a strong effect of acute SAFit1 administration on stress-induced CORT secretion, presumably due to enhanced negative feedback at the level of the pituitary gland. These data suggest that peripheral-only FKBP51 inhibition, compared to global FKBP51 inhibition, has stronger effects in reducing stress-induced CORT secretion in female rats. Because the half-life of SAFit1 is shorter than SAFit2 (2.5 versus 9 hours), there should not be any active SAFit1 at the time of the noise stress as this is 12 hours after the last dose of SAFit1. Although this is perhaps an experimental limitation it can be used to deduce the mechanism of how SAFit1 reduces stress-induced CORT. Hence the effects of SAFit1 on stress-induced CORT would be effects sustained from the administration at 19.00h the day before the noise stress. Therefore, it is plausible that the effects on stress-induced CORT by SAFit1 are due to genomic actions, presumably in the pituitary gland, rather than rapid, non-genomic actions.

Neither acute nor sub-chronic SAFit2 treatment affected stress-induced CORT. Sub-chronic SAFit2 treatment in females causes a reduction in basal CORT but had no effect on stress-induced CORT. This suggests that prolonged SAFit2 treatment enhances the pituitary gland effects of SAFit2 enough to overpower the central effects of SAFit2. Interestingly, it has been shown that in OVX female rats, DEX suppresses peak diurnal and stress-induced CORT increase. However, when OVX females were administered oestrogen replacement, the diurnal CORT concentrations increased and the suppression of diurnal CORT by DEX was abolished (401). This suggests that oestrogen inhibits negative feedback during the diurnal

peak of CORT. Moreover, the same study showed that oestrogen replacement in the OVX females significantly increased stress-induced CORT, and the DEX suppression of stress-induced CORT was abolished. Importantly these changes in CORT were accompanied by similar changes in ACTH concentrations (401) indicating that they are not due to increased activity of the adrenal gland, but rather to changes in negative feedback regulation. The same study showed that activation of ER alpha (ERα) in the PVN increased the diurnal HPA axis activity, and it was sufficient to abolish the suppressive effects of DEX during the diurnal peak in CORT. Furthermore, activation of ERα in the PVN diminished DEX suppression and results in ACTH and CORT concentrations similar to those of vehicle-treated female rats (401). Thus, these data suggest that oestradiol impairs GC negative feedback during the diurnal peak of CORT and following stress-induced CORT and that this effect is mediated by activation of ER-α in PVN neurons. It has been shown that FKBP51 is involved in TTC9A-mediated suppression of ER-α activity (402). Thus, FKBP51 inhibition may cause a reduced suppression of ER-α activity and, if this occurs in the PVN, decreased GC negative feedback. It is tempting to speculate that this could explain why SAFit2 has opposing effects in the central and peripheral, however, further investigation is required.

The female rats used in the experiments described in this chapter were not checked for the stage of their oestrous cycle. It has been shown that female rats have increased basal CORT during the proestrus stage of the oestrus cycle compared to the oestrus and metestrus stage of the cycle (232). Moreover, the same study showed that the basal CORT concentrations were similar in male rats and female rats in the oestrus stage, and the CORT concentrations were only higher in female rats compared to male rats when they were in the metestrus, proestrus, and diestrus (232). Hence the oestrus cycle stage could influence the results of an experiment involving the HPA axis activity. Previous studies using freely cycling female and male Sprague-Dawley rats found that both basal and stress-induced HPA axis activity was significantly different between the two sexes despite not controlling for cycle stage (239, 240). Hence when comparing sexual dimorphic effects of HPA axis activity it appears that controlling for oestrus cycle in the females might not be necessary. However, in this chapter vehicle-treated female rats were compared to SAFit2- or SAFit1-treated female rats and thus if the females were in different stages this would influence the HPA axis activity. Although it has for a long time been assumed that female rats that are kept together

synchronize their oestrus cycle by excreting pheromones (403), it has also been reported that this is not the case (404), and the reports seem to be mixed. Nevertheless, it is possible that the females used in the studies in this chapter were in different stages of the oestrus cycle and it would have been useful to control for this.

Other limitations include using PCR to quantify the gene expression, as discussed previously, this does not give an anatomical representation. A more suitable approach would have been ISH or RNA scope, and to include additional time points to investigate the gene expression following stress in more depth. However, as there was limited availability of the SAFit compounds is was not feasible to include more time points.

Chapter 6: Pilot studies for rat models of dysregulated HPA axis

6.1. Introduction

In the previous chapters, the results from experiments that focused on the effects of FKBP51-inhibition under normal conditions, in healthy, naïve rats have been described. However, the potential for using FKBP51-inhibition as a therapeutic tool for psychiatric illnesses in humans will involve using it in patients with a dysregulated HPA axis. Studies in primates show that increased FKBP51 expression is accompanied by GC resistance (321, 322). Further, in humans, it is indeed suggested that patients with MDD have increased FKBP51 levels (405-407). Overexpression of FKBP51 in the BLA in mice leads to increased anxiety-like behaviour, which can be mitigated by systemic administration of the FKBP51-inhibitor SAFit2 (340). Although these studies demonstrate that overexpression of FKBP51 is linked to increased HPA axis activity, no studies to date have investigated whether central FKB51 overexpression has any effect on the HPA axis ultradian pulse dynamics during 24 hours in the rat. This is an important question that would translate these findings into the clinic, as it has been shown that patients with unipolar depression have increased pulse amplitude and overall increased cortisol secretion compared to normal subjects (295, 296).

Furthermore, given the wide range of actions of GCs, including in the central nervous system, the immune system, and in metabolic processes, alterations of basal HPA axis concentrations and pulsatility can influence the susceptibility and severity of various diseases.

In this regard, the Lewis rat strain has been extensively studied for its increased susceptibility to numerous inflammatory conditions including experimentally induced arthritis (408-411) and encephalomyelitis (412, 413). In contrast, the histocompatible and closely related Fischer rat strain does not develop these conditions (409-411). The reason behind the differential responsiveness to immune stress in these two rat strains is linked to their differences in HPA axis activity. The Lewis rat shows a reduced HPA axis response to various stressors including physical (414, 415), psychological (416, 417), and immune stressors (411, 415, 417) compared to the Fischer rat. Thus it is suggested that the reduced HPA axis activity in the Lewis rat, and thus lower levels of CORT, is not able to reduce the immune and hormonal response to inflammation. Experiments using the ABS in female Fisher

and Lewis rats indicated similar CORT pulse height in the two strains, but an increased number of pulses throughout the 24-hours in the Fischer rats, resulting in higher mean basal CORT and, importantly, a lack of CORT circadian rhythm was observed (418).

To date, there have been no ABS studies characterising full 24-h CORT profiles and ultradian pulsatile dynamics of male Fischer and Lewis rats. Studies using hand sampling for CORT concentrations have showed increased morning and evening HPA axis activity in male Fischer rats compared to both Sprague-Dawley and Lewis rats. Furthermore, Lewis rats have significantly decreased CORT compared to both Sprague-Dawley and Fischer rats in the evening (419). Furthermore, FKBP51 expression in the Fischer and Lewis rats in the anterior pituitary gland and hypothalamus is currently unknown. It is an intriguing hypothesis that higher FKBP51 expression in these two areas may contribute to the increased HPA axis activity observed in Fisher rats.

The studies described in this chapter aim to investigate two potential rat models for hyperactive, or dysregulated, HPA axis activity. The first model is a virus-induced overexpression of FKBP51 (FKBP51 OE) in the PVN in male naïve Sprague-Dawley rats. The aim was to use this rat model to further elucidate whether FKBP51 regulates basal HPA axis activity. The second model is the male Fischer rat which has already been shown to exhibit increased CORT concentrations compared to Sprague-Dawley and Lewis rats, however, the ultradian CORT dynamics in this strain are unknown. Therefore, the aim was to characterise the ultradian CORT dynamics in this strain, as well as investigate if the increased HPA axis activity observed in male Fisher rats is due to changes in FKBP51 expression, compared to the Lewis rats.

6.2. Methods

6.2.1. Experiment 13: The effects of AAV-mediated overexpression of FKBP51 in the rat PVN on CORT ultradian rhythms and stress-induced CORT secretion.

It has been shown that FKBP5 expression is upregulated following acute stress (420), and that overexpression of FKBP5 in the amygdala induces anxiogenic behaviour in mice (340). The aim of this experiment was, therefore, to use the same overexpression protocol as Hartmann and colleagues to attempt FKBP51 overexpression in the PVN in rats (340).

6.2.1.1. Viral overexpression of FKBP51

Viral overexpression was performed using an adeno-associated bicistronic AAV1/2 vector (GeneDetect) as previously described (421). The vector contained a CAGHA-tagged-FKBP51-WPRE-BGH-polyA expression cassette that contained the coding sequence of human FKBP51 NCBI CCDS ID CCDS4808.1. For the control group, the same vector constructs without expression of FKBP51 (CAG-Null/Empty-WPRE-BGH-polyA) was used. Virus production, amplification, and purification were performed by GeneDetect with no restriction enzymes used. Rats were anaesthetized with isoflurane and bilaterally injected with AAV.FKBP51 or AAV.null as described below.

6.2.1.2. Bilateral PVN injection of AAV.FKBP5

Rats were anaesthetised using isoflurane mixed with oxygen, as described for cannulation surgery in section 2.4. Once total anaesthesia was achieved, the dorsal side of the head, between the ears, was shaved using an electric shaver. The rats were fixed in a stereotaxic frame with a mask for isoflurane delivery fitted to the frame. The shaved part of the head was sterilised with chlorhexidine. Using a scalpel, a small incision was made on the dorsal side of the head (approximately 1 cm) and the skin dissected to expose the skull. The scalpel was used to scrape the membrane off the skull to expose the bone surface and so that the Bregma could be located. Bregma level was identified using the stereotaxic frame and the following coordinates were used for the intra-PVN injection: Bregma -1.8 anteroposterior (AP), +/- 0.4 lateral (lat), -8.0 dorsoventral (DV). Two small burr holes were drilled through the skull, and a 10 ul, 25-G Hamilton syringe was used to deliver bilaterally 500 nl of $1.3x10^{12}$ genomic particles of AAV.FKBP51 or AAV.null using a Harvard automatic infusion pump over a 5 min period per site. The infusion was set to 100nl per minute and the needle was left in for an additional 1 minute before it was slowly withdrawn.

The skin was sutured, and wound powder applied. The rats were single-housed and allowed to recover on heat-pads. The rats were kept single-housed until the sutures had healed (5-7 days). After three weeks all rats were subjected to jugular vein cannulation as previously described (section 2.4). The rats were weighed daily to monitor their recovery and

any potential weight changes. Automated-blood sampling was conducted 5 days after the jugular vein cannulation.

Automated-blood sampling for measurement of basal CORT was conducted from 07.00h on day 1, until 07.00h the following day. At 07.00h of day 2, all rats were subject to noise stress (10 min, 96dB, white noise) and blood sampling collection continued for 60 minutes following the onset of the noise stress. Immediately after the sampling had stopped at 08.00h the rats were euthanized using pentobarbitone injection in the i.v. cannula, followed by decapitation with a guillotine. Whole brains were snap-frozen and then stored in -80°C.

6.2.1.3. Cryostat sectioning and in situ hybridisation

Frozen brains were fixed with poly freezer tissue medium (Tissue Tek O.C.T Compound, Sakura Finetek, Staufen Germany) and sectioned using a cryostat microtome at 18 µm. Brains were cut in the coronal plane through the following brain regions of interest: PVN; VMH; ARC; ventral hippocampus (VH) and NTS. Brain sections were then thaw mounted on Super Frost Plus slides (Menzel GmbH, Braunschweig, Germany) and stored at -20°C until further use.

In situ hybridization was performed using 35S labelled ribonucleotide probes for FKBP51 (forward primer: 5'CCTTGATTTCAAAGGTGAGGA; reverse primer: 5' CAGGCGTTATCCGTAGAATCA) as previously described (343). The FKBP5 Exon 9 was cloned in a pCRII-TOPO vector and the circular probe had a final concentration of 1512 ng/µl. The primers used for the linearisation were: Sense: pCRII-TOPO-T7-SISH 5'-GAATTGTAATACGACTCACTATAGGGCGAA-3', Antisense: pCRII-TOPO-Sp6-SISH 5'-CCAAGCTATTTAGGTGACACTATAGAATAC-3'.

In brief, after mixing gently by tapping the reaction tube, samples were quickly centrifuged and incubated at 37°C for 3 hours. After 1 h, another 0.5 µl of RNA polymerase was added. Unbound DNA was subsequently digested by adding 2 µl of RNAse-free DNAse I (Roche Applied Science, Mannheim, Germany) and incubated at 37°C for 15 minutes. For

purification of the riboprobe, the Qiagen RNeasy Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's protocol. The concentration of the label was measured in 2 ml of Scintillation Solution in a scintillation counter.

Prepared sections were fixed in 4 % paraformaldehyde, acetylated in 0.25 % acetic anhydride in 0.1 triethanolamine/HCL and subsequently dehydrated in increasing concentrations of ethanol (60 %, 75 %, 95 %, 100 %, Chloroform, 100 %). The antisense cRNA riboprobes were transcribed from a linearised plasmid.

Tissue sections were saturated with 100 µl of hybridisation buffer containing 3-7 x 106 CPM 35S labelled riboprobe. Brain sections were incubated overnight at 55°C. The following day, sections were rinsed in 4 X SSC (standard saline citrate), subsequently treated with RNAse A (10mg/mL) and washed in increasingly stringent SSC solutions (2x, 1x, 0.5x) at room temperature. Sections were then washed in 0.1 x SSC for 2 x 30 minutes at 64°C and dehydrated through increasing concentrations of ethanol. Slides were exposed to Kodak Biomax MR Films (Eastman Kodak Co., Rochester, NY) and developed after at least one week of exposure. Note that the cryostat sectioning and the ISH were performed by Lea-Maria Brix, Max Planck Institute of Psychiatry, Germany.

6.2.2. Experiment 14: Characterisation of FKBP5 mRNA expression in the hypothalamus and anterior pituitary gland of male Fischer and Lewis rats.

Male Fischer and Lewis rats were subject to jugular vein cannulation (see general methods, section 2.4) and allowed to recover for five days. Automated-blood sampling for measurement of basal CORT was conducted from 07.00h on day 1, until 07.00h the following day to establish CORT secretion profiles. These rats were then used for the gene expression analysis, along with the rats that had lost cannula patency and could not be used for ABS.

For the gene expression analysis, the rats were euthanized using pentobarbitone injection in the i.v. cannula, followed by decapitation with a guillotine. The brains were dissected, to isolate specific areas, and, along with the anterior pituitary gland, were snap-frozen on dry ice to be used for further gene expression analysis. All were euthanized either in the morning (AM; 08.30h to 09.30h) or in the evening (PM; 16.30h to 17.30h).

6.3. Results

6.3.1. Effects of AAV-mediated overexpression of FKBP51 in the rat PVN on CORT ultradian rhythms and stress-induced CORT secretion.

Previous studies in mice have shown that overexpression of FKBP51 in the BLA induces anxiety-like behaviour as shown by decreased entries to the open arm in the elevated plus maze and decreased time struggling in the forced swim test (340). In this study, the same viral construct was used to attempt bilateral overexpression of FKBP51 in the PVN of male rats. The hypothesis was that increased FKBP51 in the PVN would decrease GR activity and hence decrease GC negative feedback resulting in increased circulating CORT concentrations. The CORT concentrations were measured using ABS for 24 hours of basal HPA axis activity and during noise stress.

In-situ hybridisation indeed revealed overexpression of FKBP51 in the AAV.FKBP5 rats, however, the location appeared to be in the paraventricular nucleus thalami (PVT) of the thalamus rather than PVN.

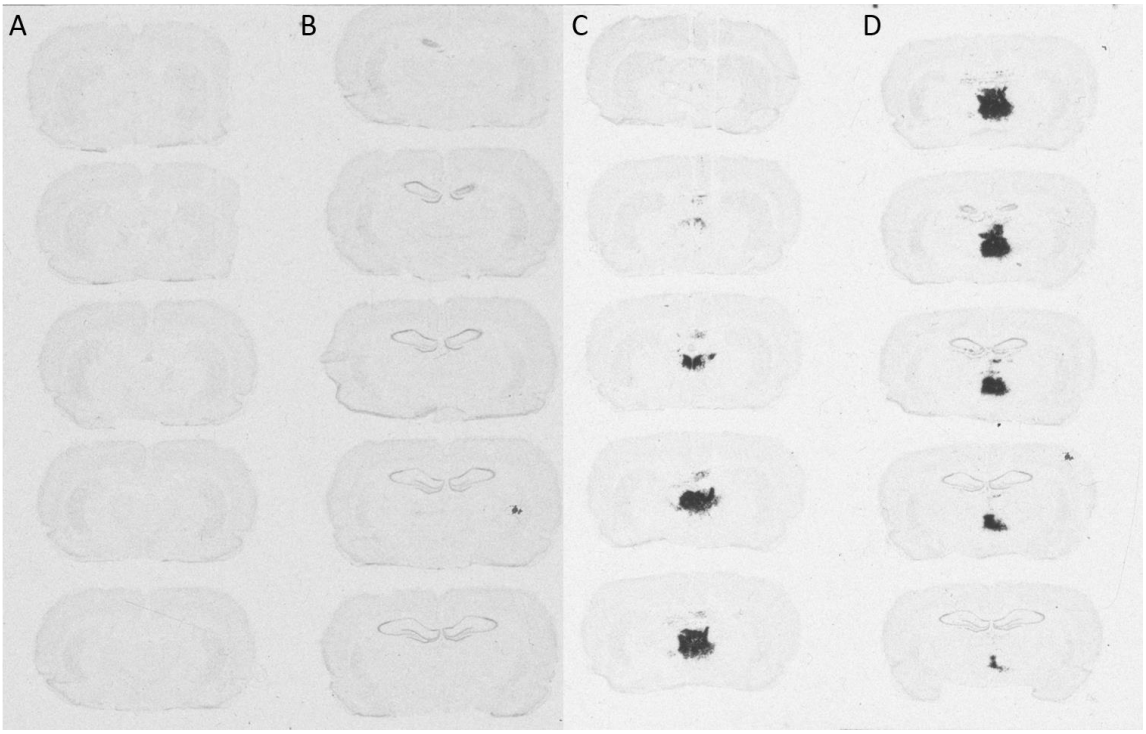

Figure 6.1. Confirmation of FKBP5 overexpression following bilateral PVN injection of AAV.null or AAV.FKBP51 in male rats. *In situ hybridisation of FKBP5 expression in two representative rats with AAV.null (A, B) and AAV.FKBP51 (C, D) bilateral PVN injection. (A) and (C) shows the FKBP5 expression at the level of the PVN in five 18 micron slices, and (B) and (D) at the level of the hippocampus in five 18 micron slices. The cryostat sectioning started at AP-1.0 and 2 series of each 8 slides, per each 5 brain slices were cut. The AAV.FKBP51 rats showed increased FKBP5 expression in the thalamus. The cryostat sectioning and in situ hybridization were performed by Lea-Maria Brix, Institute of Psychiatry, Max Planck Institute of Psychiatry, Germany.*

Although 12 rats per group received intra-cranial injections, six AAV.null and six AAV.FKBP5 rats maintained cannula patency and could be connected to the sampling system. Furthermore, one AAV.FKBP5 animal showed no overexpression and was hence excluded. Rats were all subjected to ABS every 10 minutes for 24 hours. The blood samples were assayed for CORT concentrations and the mean was calculated for each time point for both groups. The resultant average 24-h profiles are shown in figure 6.2 (A) along with the individual representative profile (B-E). All the individual 24-hour profiles were analysed with the PULSAR algorithm, and the means for each parameter are shown in figure 6.3. Further,

unpaired t-tests revealed a significant increase in the number of pulses (t(8)=-2.93, P=0.019), and the pulse frequency (t(8)=-2.93, P=0.019), in the AAV.FKBP51 rats, and a significant decrease in pulse length (t(8)=2.45, P=0.043), pulse area (t(8)=2.61, P=0.031), and pulse IPI (t(8)=2.75, 0.025), in the AAV.FKBP51 rats compared to AAV.null rats. There was no significant effect on mean CORT (t(8)=1.53, P=0.164), max CORT (t(8)=--0.28, P=0.785), basal CORT (t(8)=1.47, P=0.177), pulse amplitude (t(8)=0.62, P=0.558), pulse height (t(8)=1.01, P=0.323), basal AUC (t(8)=1.36, P=0.208), or AUC total (t(8)=1.57, P=0.153).

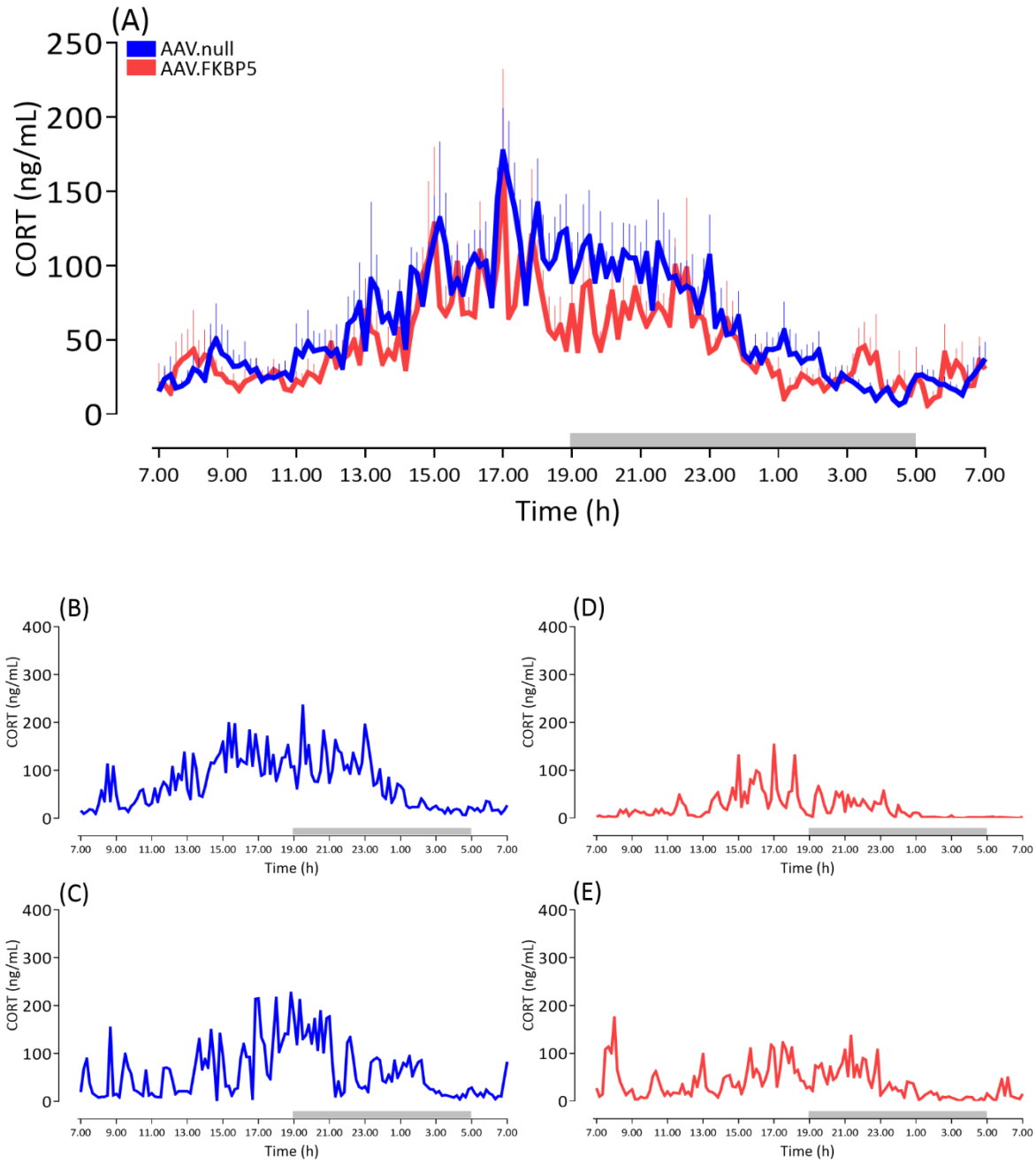

Figure 6.2. The effect of central FKBP5 overexpression on ultradian CORT secretion in male rats. *Mean + SEM (A) CORT concentrations, and representative individual profiles from male adult rats with bilateral PVN injection of AAV.null (B, C; n=6) or AAV.FKBP51 (D, E; n=6) 4 weeks prior to the experiment. Blood samples were collected via a cannula implanted in the right jugular vein every 10 min for 24-h using an ABS. Plasma corticosterone was measured by RIA. Grey bar indicates light off (19:00-05:00h). In-situ hybridisation showed the injection site was the PVT of the thalamus rather than the PVN.*

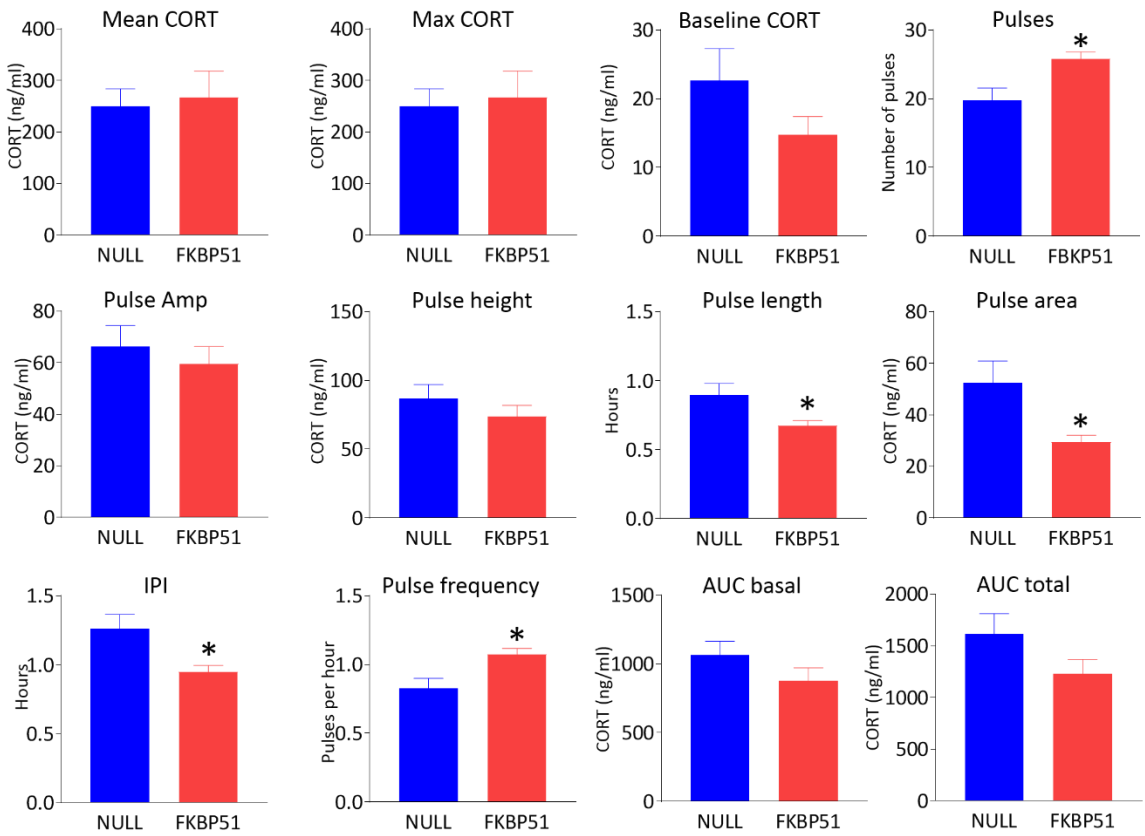

Figure 6.3. The effect of FKBP5 overexpression on PULSAR parameters in male rats. *Mean + SEM (A) CORT concentrations, and representative individual profiles from male adult rats with bilateral PVN injection of AAV.null (n=5) or AAV.FKBP51 (n=6). *P<0.05 compared to AAV.null rats, with unpaired t-test. Amp=amplitude, IPI= inter-pulse interval, AUC= area under the curve.*

These results suggest that the AAV.FKBP51 construct previously used successfully in mice (340) does indeed induce FKBP51 overexpression in rats too. However, histological analysis of the rat brains showed that the injection site targeted the thalamus, PVT to be precise, rather than the PVN. Interestingly, the data above indicate that increased FKBP51 expression in the thalamus of male rats can affect CORT ultradian pulsatility. The AAV.FKBP51 rats had an increased number of CORT pulses, (AAV.FKBP51 25.8 ±1.77; AAV.null: 19.8 ±1.01) compared to AAV.null-treated rats. Further, both the pulse length and the IPI were lower in the AAV.FKBP51 rats compared to AAV.null rats. This suggests that overexpression of FKBP51 in the thalamus of rats increases the pulse frequency but does not alter the mean CORT

concentrations. This is interesting as it suggests that thalamic GR signalling can affect CORT ultradian rhythm dynamics.

To investigate the effect of thalamic FKBP51 overexpression on the CORT response to stress, following 24-h blood sampling the rats were subjected to noise stress and blood sampling continued for 60 minutes following the noise stress (figure 6.4). Repeated measures analysis showed an overall significant effect of time ($F(1.463, 14.633)=15.730$, $P=0.001$), however no significant interaction between time and treatment ($F(1.463, 14.633)=2.340$, $P=0.603$), but a significant effect of treatment ($F(1,9)=4.544$, $P=0.020$). Further, post hoc analysis revealed a significant increase in stress-induced CORT in AAV.null rats at the 10 minutes ($P<0.0001$), 20 minute ($P=0.001$), and at the 30 minutes ($P=0.005$) time point after the noise onset, compared to AAV.null rats at the 0 minute time point. In comparison a significant increase in stress-induced CORT in the AAV.FKBP51 rats were only observed at the 10 minute time point ($P=0.018$), compared to AAV.FKBP51-treated rats at the 0 minute time point. Moreover, there was a significant difference between the AAV.FKBP51 and the AAV.null rats at the 10 minute time point ($P=0.005$). There was no difference between the baseline CORT at the 0 minute time point between AAV.null rats and AAV.FKBP51 rats.

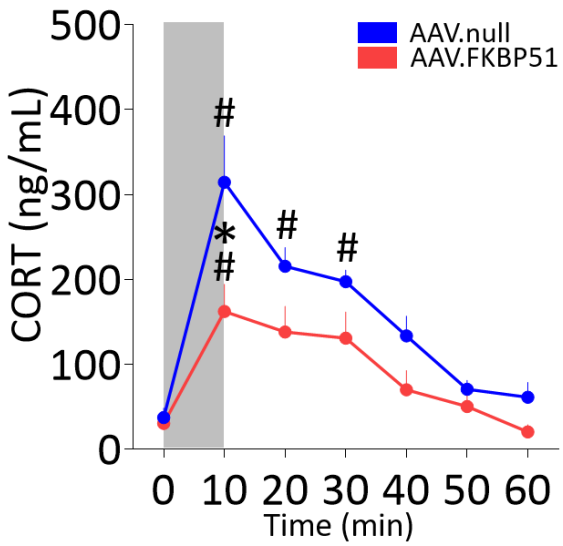

Figure 6.4. The effect of FKBP5 overexpression on stress-induced CORT in male rats. *Mean + SEM corticosterone levels during noise stress (96dB, 10 minutes, white noise, 07.00h) from male adult rats with bilateral PVN injection of AAV.null (n=6) or AAV.FKBP5 (n=7) 4 weeks prior to the experiment. Blood samples were taken every 10 minutes from a cannula implanted in the right jugular vein and CORT concentrations measured with CORT RIA. The time indicates the time after the noise stress, which was performed at 0 minutes. *P<0.05, compared to AAV.null rats with Tukey's post hoc test. #P<0.05, compared to respective time 0, with Tukey's post hoc test.*

These data suggest a trend for overall decreased CORT and a significantly shorter CORT response to noise stress in the AAV.FKBP51 rats compared to AAV.null rats.

6.3.2. Experiment 14: Characterisation of CORT ultradian rhythms, and FKBP5 mRNA expression in the hypothalamus and anterior pituitary gland, in male Fischer and Lewis rats.

Four Lewis and three Fischer male rats were all subject to ABS every 10 minutes for 24 hours and complete profiles were obtained for three Lewis and two Fischer rats. The blood samples were assayed for CORT concentrations and the mean was calculated for each time

point for both groups. The resultant average 24-h profiles are shown in figure 6.5 (A) and two individual profiles (B-E) from each group are represented.

All the individual 24-hour profiles were analysed with the PULSAR algorithm and the means for each parameter are shown in figure 6.6. However, due to the low number of rats in each group, it was not possible to perform a statistical analysis of the PULSAR data. The mean CORT, max CORT, pulse amplitude, pulse height, pulse length, pulse area, AUC basal, and AUC total were all lower in the Lewis rats compared to the Fischer rats. It should be noted that these are observations are only based on the mean in each group and not statistics. Nevertheless, these results are the first to show the full 24-hour CORT profiles of male Fischer and Lewis rats.

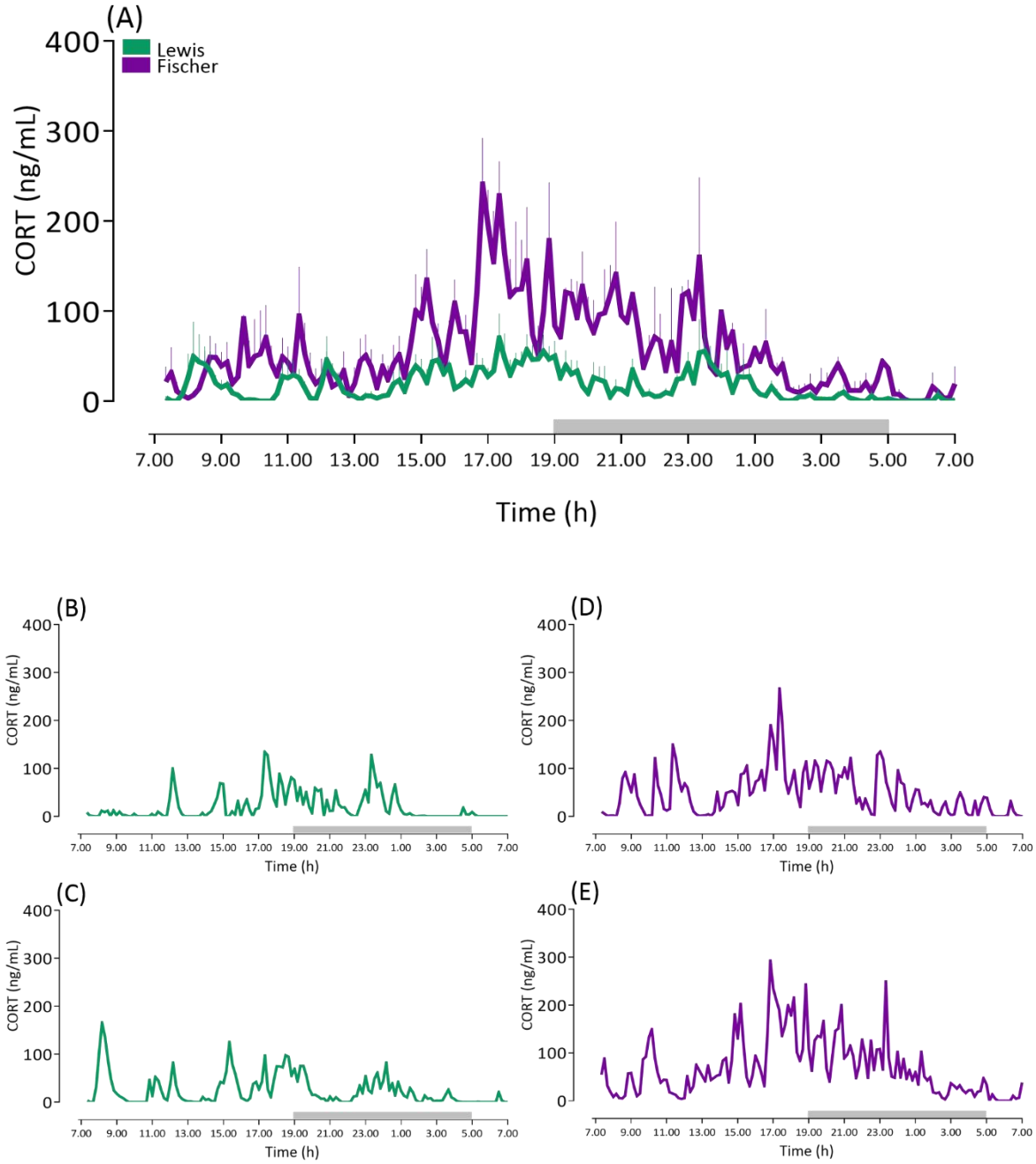

Figure 6.5. 24-hour basal CORT profiles of male Fischer and Lewis rats. *Mean + SEM CORT (A) and individual profiles from male adult Lewis (B, C; n=3) and Fischer (D, E; n=2) rats. Blood samples were collected via a cannula implanted in the right jugular vein every 10 min for 24-h using ABS. Plasma corticosterone was measured by RIA. Grey bar indicates light off (19:00 – 05.00h).*

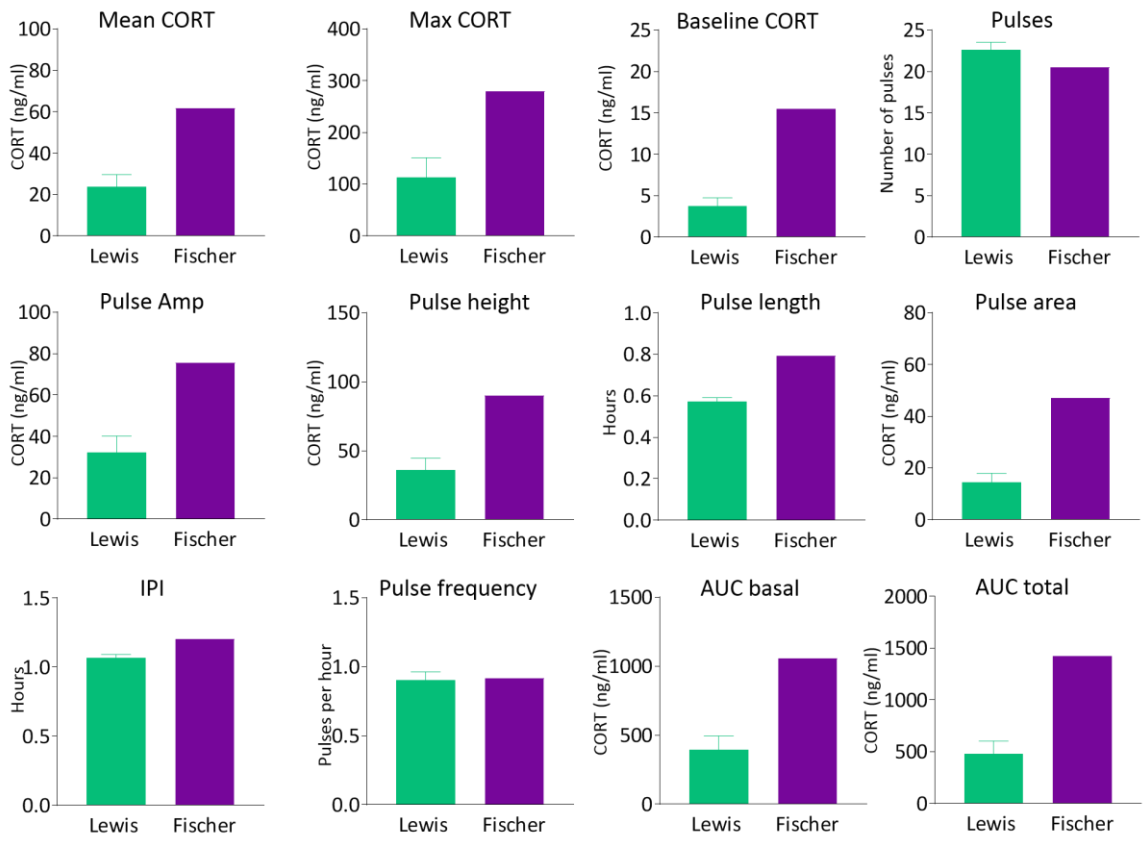

Figure 6.6. PULSAR parameters of male Fischer and Lewis rats. *Mean + SEM and mean CORT parameters calculated using PULSAR from 24-hour profiles of male adult Lewis (n=3) and Fischer (n=2) rats. Amp=amplitude, IPI= inter-pulse interval, AUC= area under the curve. Note that as there are only two Fischer rats there are no error bars for that group.*

It is currently unknown why these histocompatibility strains have such different CORT profiles. Given the pivotal role of FKBP51 in regulating GR and hence GC negative feedback, it is plausible to speculate that these differences in CORT may be due to differential expression of FKBP51 or GR. Therefore, FKBP5 and GR mRNA was quantified in the hypothalamus and anterior pituitary gland of Fischer and Lewis rats at two time points in the AM or PM. The relative expression of FKBP5 and GR mRNA in the hypothalamus in male Lewis and Fisher rats are shown in figure 6.7. For hypothalamic FKBP5 mRNA, a two-way ANOVA revealed an overall significant effect of time ($F(1, 14)=8.973$, $P=0.013$), however no significant effect of strain ($F(1, 14)=0.032$, $P=0.861$), and no significant interaction ($F(1, 14)=1.588$, $P=0.236$). Further, post hoc analysis revealed a significant increase in FKBP5 mRNA in Lewis PM rats ($P=0.037$) compared to Lewis AM rats. For hypothalamic GR mRNA, a two-way ANOVA revealed no

significant effect of time (F(1, 14)=0.009, P=0.925), no significant effect of strain (F(1, 14)=1.367, P=0.269), and no significant interaction (F(1, 14)=1.550, P=0.242). Thus, these data indicate that in the hypothalamus there is a circadian variation in FKBP5 mRNA expression in the Lewis rats, with higher expression in the PM compared to AM, whereas there is no circadian difference in the Fischer rats.

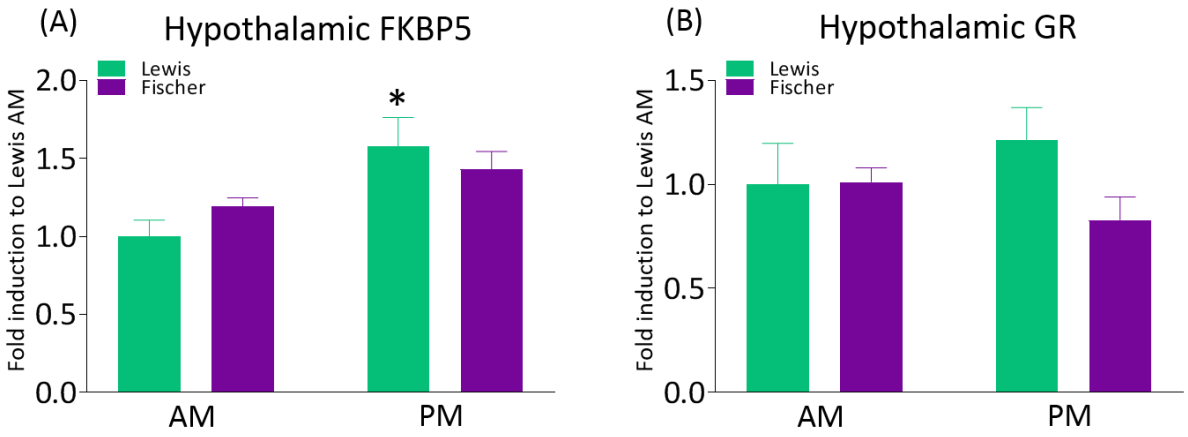

Figure 6.7. Expression of FKBP5 and GR in the hypothalamus of male Fischer and Lewis rats. *Mean + SEM FKBP5 (A) and GR (B) mRNA in the hypothalamus of male Lewis euthanized in the AM (n=4) or PM (n=4) and Fischer rats euthanized in the AM (n=3) or PM (n=3). *P<0.05, effect of time, analysed by post hoc multiple comparisons with Tukey's post hoc test.*

The relative expression of FKBP5 and GR mRNA in the anterior pituitary gland in male Lewis and Fisher rats are shown in figure 6.8. For anterior pituitary gland FKBP5 mRNA, a two-way ANOVA revealed an overall significant effect of time (F(1, 14)=62.062, P=0.00001), a significant effect of strain (F(1, 14)=38.946, P=0.0001), and a significant interaction (F(1, 14)=7.087, P=0.02). Further, post hoc analysis revealed a significant increase in FKBP5 mRNA in Lewis PM rats (P=0.012) compared to Lewis AM rats, and a significant increase in FKBP5 mRNA in Fischer PM rats (P=0.0002) compared to Fischer AM rats. In addition, there was a significant increase in FKBP5 mRNA in Fischer PM (P=0.0004) compared to Fischer AM. For anterior pituitary gland GR mRNA, two-way ANOVA revealed an overall significant effect of time (F(1,14)=15.357, P=0.003), a significant effect of strain (F(1, 14)=18.263, P=0.002),

however no significant interaction (F(1, 14)=1.550, P=0.242). Further, post hoc analysis revealed a significant increase in FKBP5 mRNA in Lewis PM rats (P=0.031) compared to Lewis AM rats, and only a trend for higher levels of FKBP5 mRNA in Fischer PM rats (P=0.092) compared to Fischer AM rats. Furthermore, there was a significantly higher levels of FKBP5 mRNA in Fischer AM (P=0.030) compared to Lewis AM.

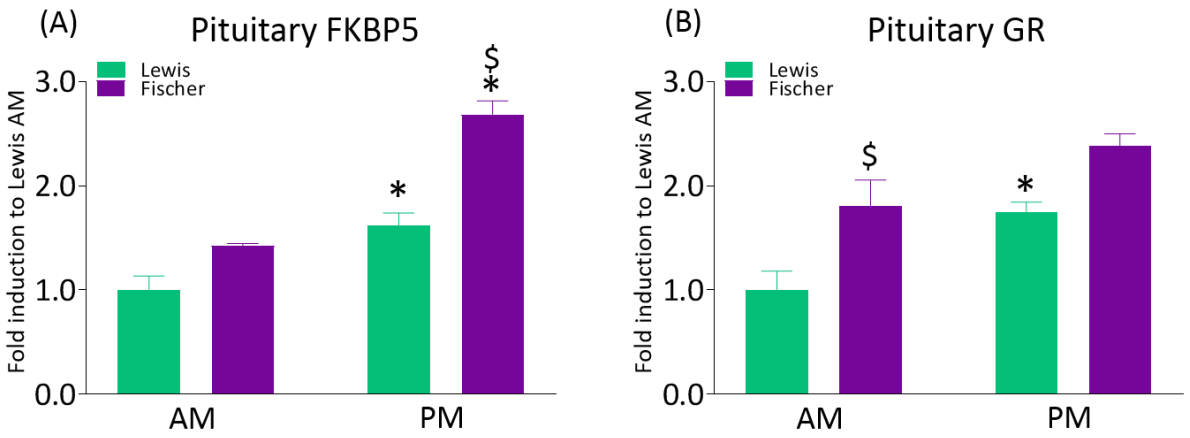

Figure 6.8. Expression of FKBP5 and GR in the anterior pituitary gland of male Fischer and Lewis rats. *Mean + SEM FKBP5 (A) and GR (B) mRNA in the anterior pituitary gland of male Lewis euthanized in the AM (n=4) or PM (n=4) and Fischer rats euthanized in the AM (n=3) or PM (n=3). *P<0.05, effect of time, analysed by post hoc multiple comparisons with Tukey's test. $P<0.05, effect of strain, analysed by post hoc multiple comparisons with Tukey's post hoc test.*

These data indeed demonstrate differential expression of both FKBP5 and GR in the anterior pituitary gland in Lewis and Fischer rats. The most noticeable finding is the increased FKBP5 mRNA expression in the Fischer rats in the PM, compared to the Lewis rats in the PM.

6.4. Discussion

This chapter contains preliminary data from two rat models of dysregulated HPA axis activity suggesting that these rats could potentially be used to further investigate the role of FKBP51 in the regulation of HPA axis activity and CORT ultradian rhythm.

6.4.1. FKBP51 overexpression

Little is known about the role of FKBP51 in the thalamus, and only a few studies have investigated the role of the thalamus in regulating HPA axis activity. Studies have shown that some areas of the thalamus, including the lateral parvocellular region of the subparafascicular nucleus and the posterior intralaminar nucleus, project directly to CRH-containing neurones in the PVN (361). In particular, these thalamic regions seem to be sensitive to auditory stress (361) such as the noise stress used in the present study. Other studies have shown that in the rat the PVT has strong projections to the CeA, BLA, and BNST (22-24), areas that are implicated in the regulation of HPA axis activity (149). Thus, the thalamus has indeed been shown to have a role in HPA axis regulation.

Interestingly, in the above study, there was a strong effect on CORT pulsatility following thalamic FKBP51 overexpression, with increased pulse frequency in the AAV.FKBP51 rats compared to AAV.null rats. According to the mathematical model of ultradian CORT pulsatility developed by Walker and colleagues, an increase in CRH drive causes an increase in ultradian CORT pulse frequency (132). Overexpression of FKBP51 in the BLA, using the same construct as in the present study, showed an increase in anxiety-like behaviour in mice (340) and this is not surprising, as the amygdala has an excitatory effect on HPA axis activity (149). How the FKBP51 is involved in this thalamus-mediated change in HPA axis pulsatility is yet to be unravelled. Lesion of the medial geniculate nucleus of the thalamus auditory system has been shown to block CORT secretion following audiogenic stress in rats (422). In the present study stress-induced CORT following noise stress was lower in the AAV.FKBP51 rats suggesting that FKBP51 overexpression and the potential reduction in nuclear GR in the thalamus is involved in the audiogenic stress regulation, although this has not been tested.

6.4.2. Fisher and Lewis rats

The 24-hour CORT profiles of the Lewis and Fischer male adult rats differ from the previously published female Lewis and Fischer CORT profiles. Female Fisher rats exhibit increased CORT pulse amplitude throughout the 24-hour and hence lack a circadian variation in mean CORT (418). In contrast, the male Fischer rats in the present study showed a circadian variation in CORT pulse amplitude, however, the overall CORT concentrations were higher compared to the male Lewis rats. Observations from the PULSAR analysis suggests the increased CORT in the Fischer rats is due to increased pulse amplitude and pulse height. This increase in CORT in the male Fischer rats, compared to Lewis rats, is consistent with previous reports (419). The results from the gene expression analysis suggest that the higher CORT concentrations in the Fischer rats, compared to Lewis rats, is at least partly due to higher expression of FKBP51 in the Fischer rats. The Fischer rats had significantly higher FKBP51 mRNA expression in the PM group compared to Lewis rats in the PM group. It is plausible that this causes decreased GR transcriptional activity, decreased GC negative feedback, and a subsequent increase in CORT. The increased GR mRNA in the Fischer AM compared to Lewis AM, might mitigate these effects in the morning and could explain why there is a circadian variation in CORT. In light of the data showing sexual dimorphism in the effects of SAFit1 and SAFit2, it would be interesting to determine the expression profiles of FKBP51 and GR in female Fischer and Lewis rats. Perhaps female Fischer rats have no circadian variation in the GR expression and hence why there is no circadian variation in CORT concentrations.

In this chapter, data has been presented from two novel rat models of dysregulated HPA axis function, which could both be useful for further characterisation of the involvement of the FKBP51 in HPA axis regulation and novel therapeutics. For example, it would be interesting to characterise the potential effects of SAFit2 and SAFit1 in Fischer and Lewis rats and to investigate whether this would mitigate the increased FKBP51 expression in the Fischer rats. In addition, it would be of interest to investigate whether SAFit2 would counteract the overexpression of FKBP5 in the PVT. As the viral vector of FKBP5 overexpression used in this study did indeed work, it will also be interesting to characterise the effect of FKBP5 overexpression in other brain areas, for example, the PVN, hippocampus, and the amygdala.

Chapter 7: General Discussion

7.1. Introductory statement

Although a vast amount of research is being done to further elucidate the molecular mechanisms responsible for psychiatric disorders such as major depression, bipolar disorder, schizophrenia, and anxiety disorders (71, 137), those mechanisms are not fully understood. However, it has been shown that a dysfunctional HPA axis activity is often present in patients with psychiatric disorders. This can, in turn, lead to a failure to terminate the cortisol response to stress, thus creating stress-hypersensitivity. Studies have found that this HPA axis dysfunction is partly due to a decreased activity of the GR (71), which results in an inadequate GC negative feedback response and increased cortisol concentrations (137).

What causes this dysfunction of the GR? Recent studies found that certain variants of SNPs in the gene encoding for FKBP51 (FKBP5) is correlated with hyperactivity of the HPA axis in depressed patients and with the effectiveness of antidepressant treatment in these patients (334). Because of its role in regulating GC negative feedback via modulation of GR activity, the general hypothesis is that increased expression of FKBP51 will cause a decrease in transcriptional GR activity, followed by decreased GC negative feedback and an increase in circulating GC. This led to the intriguing hypothesis that inhibition of FKBP51 could represent a valid therapeutic tool to treat depression associated with HPA axis hyperactivity. For many years, the development of a selective FKBP51-antagonist has been hindered by the structural similarity between FKBP51 and FKBP52. A recent breakthrough came when highly specific FKBP51-antagonists, SAFit1 and SAFit2, were developed by Professor Felix Hausch at the Max Plank Institute of Psychiatry in Munich (347). Since then, SAFit1 and SAFit2 have been experimentally used in mice to characterise the effects of FKBP51-inhibition on HPA axis activity (347), as well as its effect on anxiety-like behaviour (340), metabolism and pain (351, 423). Within this thesis, the effects of SAFit2 and SAFit1, on basal and stress-induced CORT secretion, in both male and female rats have been investigated. The results of the experiments have extended our previous knowledge on the regulation of GC-mediated negative feedback and highlighted the role of FKBP51 in

regulating the HPA axis and have further elucidated the sexual dimorphism in HPA axis activity.

7.2. Summary of aims and findings

Table 7.1. *Summary of the main findings from the SAFit2 and SAFit1 experiments. HY= hypothalamus, PIT= anterior pituitary gland.*

	SAFit2 male acute	SAFit2 male sub-chronic	SAFit1 male acute	SAFit1 male sub-chronic	SAFit2 female acute	SAFit2 female sub-chronic	SAFit1 female acute
Basal CORT	↓	↓↓	-	↓	-	↓	↓
Stress CORT	↓	↓↓	-	↓	-	-	↓
GR inducible gene expression	-	↑ HY	-	-	-	↑ PIT	↑ PIT

The general aim of this project was to characterise the role of the GR chaperone protein FKBP51 in the regulation of HPA axis activity and corticosterone ultradian rhythm in the rat. The overall hypothesis was that by inhibiting FKBP51, GR activity will be altered, and this will have profound effects on GC negative feedback. The most important finding of this research is that treatment with the central and peripheral FKBP51-inhibitor SAFit2 for five consecutive days caused a reduction in basal CORT secretion in both male (figure 4.2) and female rats (figure 5.3), whereas a reduction in stress-induced CORT was observed only in male rats (figure 4.6) and not in the female rats (figure 5.7). Furthermore, treatment with the peripheral-only FKBP51 inhibitor, SAFit1 caused changes in ultradian pulse dynamics in male rats (figure 4.5). The molecular mechanisms underlying the observed effects are yet to be elucidated however following prolonged SAFit2 treatment a significant increase in GR-inducible genes was observed in the hypothalamus in male rats (figure 4.7), and in the anterior pituitary gland in female rats (figure 5.13). The summary of the main effect following SAFit2 and SAFit1 administration in male and female rats are shown in table 7.1. The data produced by this work will help elucidate the role of the GR chaperone, FKBP51, in the regulation of HPA axis activity.

In this chapter the results will be discussed in relation to the following aims:

Aim 1. To elucidate the effects of SAFit2 and SAFit1 treatment on the HPA axis activity in male rats.

Aim 2. To elucidate the effects of SAFit2 and SAFit1 treatment on the HPA axis activity in female rats.

Aim 3. To elucidate the molecular mechanisms underlying the effects of SAFit2 and SAFit1 on the HPA axis activity described in aim 1 and 2.

7.3. Effects of SAFit2 and SAFit1 treatment on the HPA axis activity in male rats

The first aim of the project was to characterise the effects of two FKBP51-inhibitors, SAFit2 and SAFit1, on basal and stress-induced CORT concentrations, and on the ultradian rhythm of CORT secretion in male rats. The hypothesis was that by inhibiting the activity of FKBP51, SAFit2, and SAFit1 would enhance GR nuclear translocation and therefore affect its transcriptional activity. This would subsequently enhance GC negative feedback and ultimately reduce CORT secretion. Since SAFit2 can block FKBP51 expression both peripherally and centrally, whereas SAFit1 affects only peripheral FKBP51, the hypothesis was that the effects of these two compounds would be different. Indeed, the results demonstrate a reduction in both maximum CORT, and stress-induced CORT secretion following acute SAFit2 administration in male rats (figure 3.3 and 3.6). In contrast, there was no effect on either basal or stress-induced CORT following acute SAFit1 administration in male rats.

Within this same aim, the effects of prolonged exposure to SAFit2 and SAFit1 on basal and stress-induced CORT in male rats was investigated. The FKBP51 inhibitors have been suggested to represent a novel therapeutic tool for the treatment of mental illnesses linked to a dysregulated HPA axis activity. Therefore, the sub-chronic administration of SAFit2 and SAFit1 was performed to better understand the effects of prolonged treatment with an FKBP51 inhibitor. Following sub-chronic treatment with SAFit2 or SAFit1, both inhibitors significantly lowered stress-induced CORT concentrations in male rats (figure 4.2 and 4.4). This contrasts with the results from the acute studies where only the SAFit2 caused lower stress-induced CORT concentrations. This data, therefore, suggests that an acute increase in

pituitary gland GR-mediated negative feedback may not be sufficient to reduce stress-induced CORT, however, when rats are exposed to the prolonged enhancement of pituitary gland-mediated GC negative feedback, these changes can lead to reduced stress-induced CORT secretion.

Further, the data show that sub-chronic SAFit1 treatment alters the ultradian rhythm of CORT secretion in male rats. By analysing the 24-hour CORT profiles with the PULSAR algorithm the data revealed that in the male SAFit1 group the number of pulses increased and both the pulse length and IPI were shorter, which resulted in a smaller pulse area (figure 4.5). In contrast, sub-chronic SAFit2 administration resulted in lower maximum and mean CORT concentration, pulse amplitude and height, and CORT 24-h AUC, but no effects on the dynamic of CORT pulses (i.e. pulse length and IPI) were found (figure 4.3). Thus, the data suggest that prolonged peripheral-only inhibition of FKBP51 alters ultradian CORT pulsatility dynamics in male rats, while central and peripheral inhibition reduces the pulse height and subsequently the mean and max CORT concentrations, with no effects on the dynamic of the CORT pulses.

Finally, the aim was to characterise at what level of the HPA axis SAFit2 and SAFit1 are acting. In other words, to investigate how the central and peripheral regulation differs and what effects this produces to obtain a better understanding of how the HPA axis is regulated by FKBP51. Indeed, determining if SAFit2 and SAFit1 are causing their effects at the level of the hypothalamus or other brain areas (SAFit2 only), at the level of the pituitary gland, or in the adrenal gland directly, could help characterise the mechanism of FKBP51 involvement in the GC negative feedback. To investigate the effects of the FKBP51 antagonists at the three different levels of the HPA axis, the following three models were used to activate HPA axis activity, pituitary-adrenal gland activity and/or adrenal gland CORT secretion, respectively: i) noise stress, ii) CRH injection, iii) ACTH injection (chapter 3). Performing these experiments allowed for determining whether there was any difference in the responses to each stimulation model, and hence if the SAFit2 and SAFit1 affected any specific level of the HPA axis.

Following the CRH injection, both SAFit2- and SAFit1-treated rats had lower ACTH secretion compared to the vehicle-treated rats (figure 3.9). The CORT concentrations measured at the same time points were also lower in both the SAFit2- and SAFit1-treated rats

compared to vehicle-treated rats (figure 3.10). Thus, suggesting that both SAFit2 and SAFit1 reduce the CORT response following a CRH injection by reducing ACTH secretion. Furthermore, neither SAFit2 nor SAFit1 had any effect on ACTH-induced CORT secretion (figure 3.11), indicating that there is no direct effect of SAFit2 or SAFit1 on the adrenal glands. These experiments indicate that lower CORT concentrations following CRH administration in both the SAFit2 and the SAFit1 group are due to lower ACTH secretion from the anterior pituitary gland.

7.3.1. Effects of SAFit2 and SAFit1 treatment on basal and stress-induced corticosterone secretion in male rats

Acute SAFit2 administration decreased max CORT concentrations under basal conditions while there was no effect of acute SAFit1 administration. Furthermore, the CRH and ACTH injection experiments (chapter 3), as mentioned above, suggest both SAFit2 and SAFit1 are acting in the anterior pituitary gland to reduce ACTH secretion. However, only acute SAFit2 treatment results in lower stress-induced CORT, whilst there was no effect of acute SAFit1 treatment (figure 3.6).

Therefore, as both SAFit2 and SAFit1 are active in the anterior pituitary gland, this suggests that SAFit2 must have additional effects elsewhere to produce the observed decrease in CORT that was not seen in the SAFit1-treated rats. As SAFit2 should cross the BBB, it is plausible that SAFit2 is, in addition to the pituitary gland, also acts centrally to reduce CRH secretion following a stressor. However, if SAFit1 reduces the ACTH and subsequent CORT response to exogenous CRH, why is there no reduction in CORT following stimulation with endogenous CRH, such as that induced by noise stress? The CORT concentrations following the noise stress were approximately two-fold higher than following exogenous CRH stimulation. As only SAFit2 caused a reduction in stress-induced CORT concentrations, it is plausible that when the rats are exposed to higher concentrations of CRH, a central enhancement in negative feedback is required to lower the stress-response. Enhanced negative feedback at a central level would reduce CRH concentrations. In contrast, enhanced negative feedback in the pituitary gland would not affect CRH concentrations, and thus in the presence of high CRH levels, perhaps enhanced negative feedback in the pituitary gland is not

sufficient to reduce ACTH and subsequent CORT secretion. To investigate whether SAfit2 directly affects CRH synthesis/secretion, it would have been interesting to administer SAFit2, expose the rats to noise stress, terminate the rats at different time points and perform in situ hybridisation for CRH mRNA and/or hnRNA levels in the PVN, and POMC in the anterior pituitary gland.

It is known that rapid, GC-induced negative feedback is induced both centrally, for example in the PVN, hippocampus, and amygdala, and also in the pituitary gland (357). In the hypothalamus, this rapid negative feedback is thought to depend on endocannabinoid signalling involving the CB1 (143). For example, CB1 knock out mice display a greater stress-response to the forced swim test and tail suspension test (424, 425). When GCs are administered systemically, the HPA axis activity is rapidly reduced (172, 426). To determine whether this rapid negative feedback is occurring in the PVN, Evanson and colleagues performed stereotaxic injections of GCs bilaterally in the PVN. The result indeed indicated a rapid suppression of HPA axis activity following an acute stressor (143). Furthermore, this rapid negative feedback was also observed in rats injected with DEX conjugated to BSA, and hence unable to cross the plasma membrane (143). Thus, these data suggest that rapid negative feedback in the PVN is dependent on a putative membrane-associated GR and endocannabinoid signalling.

Nevertheless, the anterior pituitary gland has also been implicated in the rapid, stress-induced GC negative feedback mechanism (427, 428). For example, HPA activation by CRH injection is attenuated by pre-treatment with GCs, and this is GR-mediated (428, 429). Indeed, Cole and colleagues showed that the CORT response to a CRH challenge was completely abolished when the rats were pre-treated with DEX. However, DEX pre-treatment had no effect when the rats were subject to an ACTH-challenge (428), indicating the rapid, GC-induced negative feedback occurred in the anterior pituitary gland and was GR-mediated. Furthermore, human studies have shown that prednisolone (a GR agonist treatment caused reduced ACTH and cortisol concentrations following CRH stimulation, and these effects were observed within 30 minutes of CRH administration (429). Similarly, in the rat, the administration of methylprednisolone induces a rapid suppression of CORT secretion both in male (379) and in female rats (430).

The noise stress used in the acute and sub-chronic experiments was performed at 07.00h when the HPA axis activity in rats is low (see for example figure 3.2) and there was no visible difference in basal CORT concentrations before the noise stress between vehicle and SAFit1- or SAFit2-treated rats. Therefore, if the SAFit2- or SAFit1-treated rats show a blunted CORT response to the noise stress this is not attributed to a difference in the basal CORT concentrations before the noise, but rather an enhancement in the rapid GC negative feedback in response to the stress. As GC-mediated rapid negative feedback in the PVN, hippocampus, and amygdala have shown to be GR-dependent (357), perhaps the inhibition of FKBP51 can regulate this rapid negative feedback. Therefore, it is reasonable to suggest that acute SAFit2 treatment enhances rapid GC-induced negative feedback centrally. The exact mechanism is beyond the scope of this thesis, however this putative membrane-associated GR shares homology with the intracellular GR (431) and it is therefore likely to bind FKBP51. The FKBP51 impairs ligand (CORT) binding to the GR (432) and thus FKBP51 inhibition by SAFit2 could enhance CORT-mediated activation of the putative membrane-associated GR and therefore enhance rapid, endocannabinoid-mediated GC negative feedback in the PVN.

In the sub-chronic studies, SAFit2-treated male rats showed decreased mean and maximum CORT, and stress-induced CORT, compared to the vehicle-treated rats (figure 4.3 and 4.6). As suggested above, the reduction in basal and stress-induced CORT following both acute and sub-chronic SAFit2 treatment may be due to enhanced GC negative feedback in the PVN, or other brain areas involved in regulating the HPA axis such as the hippocampus or amygdala.

Rapid negative feedback does not only occur centrally but also at the level of the anterior pituitary gland. In the corticotrophs of the anterior pituitary gland, CRH stimulates the increase in bursts and frequency of action potentials (200) which in turn increases intracellular calcium and vesicle-mediated release of ACTH (201, 202). This increase in burst-type firing is dependent on the activity of BK channels (200, 204) suggesting that the BK channels are required for ACTH release. It has been shown *in vitro* that CORT reduces both spontaneous and CRH-mediated burst-type firing and that this is BK channel-dependent (433). The exact mechanism is not yet clear, however, it has been suggested that CORT is involved in BK channel deactivation by phosphorylation (206, 207). Furthermore, it has been shown that the rapid effects of GCs on the BK channel regulation in the corticotrophs depend on

activated intracellular GR (434, 435). In addition to this, it has been demonstrated that the protein ANXA1 is involved in the regulation of ACTH secretion from the corticotropic cells, and this involves reorganisation of the actin cytoskeleton (208). This has been proposed to be dependent on the GR activation and a subsequent kinase cascade (209). It has been shown that the stress-induced effects of GCs on ACTH and CORT inhibition can me mimicked by intracerebroventricular (i.c.v.) injections of human recombinant ANXA1 (hrANXA1) *in vivo*. It is suggested that ANXA1 inhibits ACTH release from the pituitary gland by inhibiting the exocytosis of ACTH (208). This process is dependent on GR activity in the folliculostellate cells, where GC initiates GR activation, which in turn activates a kinase cascade, and ANXA1 translocates to the plasma membrane where it can be secreted to nearby corticotrophs (209). It is not yet clear if FKBP51 inhibition would have any effect on this process, however, it has been shown that when FKBP51 is bound to the GR-Hsp complex this reduces the affinity of the receptor complex for CORT (315, 316). Thus, SAFit2 and SAFit1 may inhibit FKBP51 bound to GR, which allows CORT to bind and activate GR to allow for the kinase cascade and ANXA1 secretion to inhibit ACTH. Although these are only speculations it would be interesting to test this experimentally.

Thus, there are several different mechanisms by which a change in GR activity can affect ACTH secretion from corticotrophs in the anterior pituitary gland. FKBP51 regulates GR translocation to the nucleus (72) and hence the transcriptional activity of GR. Besides, FKBP51 also regulates the steroid affinity of GR, as when FKBP51 is bound to the receptor complex the affinity for GCs is reduced (436). Furthermore, FKBP51 has also been shown to regulate the activity of several kinases such as AKT (366) and GSK3B (328), both of which regulate GR activity (367). Inhibition of FKBP51 in the anterior pituitary gland by SAFit2 or SAFit1 may, therefore, decrease ACTH release in various ways following stress-induced increases in CORT secretion.

Why is the reduced stress-induced CORT not seen in the acute SAFit1 experiment? It is clear from the data that prolonged treatment is required to observe a significant effect of SAFit1 on HPA axis activity. Similar results were observed in the SAFit2 experiments, where the reduction in stress-induced CORT was further enhanced with sub-chronic SAFit2-treatment in males compared to the acute SAFit2-treatment (figure 3.6 and figure 4.6). In the sub-chronic SAFit2-treated males, the effect on the 24-hour CORT was also enhanced,

compared to the acute treatment experiment (compare figure 3.2 with figure 4.2). Thus, this suggests that prolonged treatment with the SAFit2 or SAFit1 augments (SAFit2) or induces (SAFit1) their effects on HPA axis activity. In comparison, a previous study using a GR antagonist, Org34850, measured the 24-hour CORT secretion in adult male rats (352). Whereas acute treatment did not affect either basal or stress-induced CORT secretion, 5 days of consecutive treatment with Org34850 resulted in overall increased basal CORT concentrations (353). Moreover, there was no effect of sub-chronic Org348540 treatment on stress-induced CORT secretion following noise stress (353). Org34850 occupies GR in the hypothalamus, frontal cortex, and pituitary gland but fails to occupy GR in the hippocampus, amygdala, and neocortex (437). The effects on basal CORT secretion of SAFit2 and Org34850 are somewhat opposite, which is consistent with the fact that SAFit2 should enhance GR activity and Org34850 reduces GR activity. It is unclear why Org34850 does not have any effect on stress-induced CORT, however, a separate mechanism may regulate stress-induced GC-negative feedback, compared to basal GC-negative feedback. SAFit2 administration did, however, affect the stress-induced CORT response. The SAFit2 is presumably active in several brain areas, and perhaps has more widespread effects than Org34850.

7.3.2. Effects of SAFit2 and SAFit1 on gene expression in male rats

In the hypothalamus, there was a trend of increased c-Fos mRNA expression following noise stress in the sub-chronic SAFit2 group (figure 4.7 A), and significantly decreased c-Fos mRNA expression in the sub-chronic SAFit1 group compared to the respective vehicle-treated group (figure 4.7 B). As c-Fos is a marker of cellular activity (378), this suggests that SAFit2 increases and SAFit1 decreases hypothalamic neuronal activity in response to stress. It has been shown that c-Fos expression is induced following audiogenic stress in most of the hypothalamic nuclei including the PVN (361). Since the sub-chronic SAFit2 treatment lowered the stress response, it could be expected that the c-Fos expression would also be lower, however, the results indicated a trend towards higher c-Fos expression compared to vehicle-treated rats. In contrast, in the sub-chronic SAFit1 group, where there was also a lower stress-response, the c-Fos expression was significantly lower. From this, it can be deduced that measuring c-Fos expression in whole hypothalamic extract, and quantifying the expression by RT-qPCR does not reveal much information regarding which neurones are activated. A more

suitable technique would be, for example, ISH or immunocytochemistry for Fos protein which would give an anatomical quantification of c-Fos/Fos expression. For example, one study investigated c-Fos expression in various brain areas following 30 minutes of constant noise stress (361). The rats were euthanised immediately following the noise stress and ISH used to investigate the induction of c-Fos in the brain (422). The group found that a robust increase in c-Fos mRNA was observed following acute noise stress in the majority of the brain, including the cortex, hypothalamus, thalamus, and hippocampus (422). Perhaps a more robust change in c-Fos expression would have been present if more time points and a different technique such as ISH had been used.

Interestingly, in the males, an increase in GR-responsive genes expression was only seen following sub-chronic treatment with SAFit2. In the hypothalamus of SAFit2-treated rats, both GILZ and SGK1 mRNA expression were significantly higher compared to vehicle-treated rats (figure 4.7 C). This is particularly interesting as the stress-induced CORT concentrations were significantly lower in the sub-chronic SAFit2-treated rats, and hence the activation of GR by CORT is lower than in the vehicle-treated rats. This suggests that sub-chronic treatment with SAFit2 does induce enhanced GR transcriptional activity, which in turn either enhances the activity of nuclear GR or increases the translocation of GR to the nucleus. There was no increase in GR-inducible genes in the sub-chronic SAFit1-treated males, which is consistent with SAFit1 not being active centrally.

Furthermore, in the anterior pituitary gland of the sub-chronically treated animals, there was no increased expression of GR-inducible genes in the SAFit2-treated male rats compared to the vehicle-treated male rats (figure 4.8 C). Similarly, no increase in GR-inducible genes was observed in the sub-chronic SAFit1-treated male rats (figure 4.8 D). Results from the CRH-injection study showed that both SAFit2 and SAFit1 act in the anterior pituitary gland to reduce ACTH concentrations following CRH stimulation. The lack of induction of GR-inducible genes in the anterior pituitary gland in both the SAFit2- and SAFit1-treated males suggest that the effect may be non-genomic. As mentioned (see sections 1.5, 1.6.1, 1.7.1) there are various non-genomic actions of GR, some of which are directly related to the negative feedback mechanisms in the anterior pituitary gland including GC-mediated effects on BK channels (200) and ANXA1 (208), as discussed above.

Another study investigated the induction of gene expression of various genes in the dentate gyrus, the CA regions, and in the ventral hippocampus following FST for 15 minutes in 25°C water. The rats were sacrificed immediately following the FST, or 30, 60, or 180 minutes following the onset of the FST (355). The study found that FKBP5 mRNA was only increased at 180 minutes post-stress, while both SGK1 and PER1 mRNA were both increased at 60 minutes post-stress. Moreover, the same study measured the hnRNA expression of the same genes and found a robust increase in SGK1 and PER1 hnRNA at the 30 minute time point, and at the 60 minute time point for FKBP5 hnRNA (354). This study highlights the importance of measuring gene expression at multiple time points post-stress. An increased number of time points would have been ideal, but this was not feasible due to a limited amount of SAFit2 and SAFit1 available.

7.3.3. Acute versus sub-chronic effects of SAFit2 and SAFit1 in male rats

Acute treatment with SAFit2 caused a decrease only in max CORT and, following the sub-chronic SAFit2 treatment, the overall 24-hour CORT secretion was decreased due to lower CORT pulse amplitude (figure 3.3 and figure 4.3). In comparison, acute SAFit1 treatment did not have any effects on basal CORT, and sub-chronic SAFit1 treatment did not affect the amplitude of the CORT pulses but did increase their frequency (figure 3.5 and 4.5). This suggests that acute treatment with SAFit1 is not sufficient to exert any effects on basal HPA axis activity, effects are however observed following sub-chronic treatment. This is consistent with the SAFit2 studies: longer treatment renders a greater effect. This is also consistent with previous studies using a GR antagonist, where prolonged treatment had a greater effect on HPA axis activity (353, 437).

Importantly, sub-chronic treatment with SAFit1, in contrast to SAFit2, does alter the dynamics of ultradian CORT pulses, without affecting the overall CORT concentration (figure 4.5).

7.3.4. Changes in pulsatility following sub-chronic SAFit1 treatment in male rats

Walker and colleagues (131, 132) have described a mathematical model of CORT regulation that suggests CORT pulses are independent of the CRH pattern but are governed by a feed-back/feed forward dynamic within the pituitary-adrenal gland system (figure 1.3). Therefore, when the CRH drive is low, as observed in the rat under basal conditions, CORT secretion is within a 'pulsatile' region. In contrast, when the CRH drive is higher, for example following stress, CORT secretion is in the 'constant' region and the CORT pulsatility is governed by the pituitary-adrenal gland system and by the frequency of CRH forcing (CRH drive). This indicates that when CRH concentrations are within a physiological range even constant CRH infusion will generate a pulsatile ACTH and CORT response. Indeed, this was demonstrated by Walker and colleagues *in vivo* by infusing rats with three different doses of CRH at a constant pattern and measuring the CORT output (131). At lower physiological doses of constant CRH, the subsequent CORT response was pulsatile. Moreover, this pulsatile response was similar to that of rats with endogenously regulated CRH secretion. When the rats were exposed to a higher dose of constant CRH the CORT response was also constant (131).

This model of pituitary-adrenal gland regulation of ultradian pulsatility is consistent with the changes in pulse dynamics following sub-chronic SAFit1 treatment (figure 4.5). The SAFit1 is only active peripherally and exerts HPA axis effects by interfering with the negative feedback in the pituitary gland. Hence, there are no changes to the central regulation of CRH secretion. There has been a longstanding notion that CORT pulsatility is under hypothalamic regulation, like other pulsatile hormones such as LH and GH, whose pulsatility depends on their respective hypothalamic release factor (125-127). However, in rats CRH has a pulse frequency of 3 pulses per hour (114), compared to approximately 1 pulse per hour for ACTH and CORT (102), hence there is a mismatch between the hypothalamic release of CRH and ACTH and subsequent CORT release. The fact that SAFit1 alters pulse frequency is a novel finding, and further evidence for the model of a pituitary-adrenal gland feed-forward/feedback loop of CORT pulsatility.

Furthermore, the mechanism behind this model of CORT pulsatility is dependent on ACTH-mediated positive feedforward drive from the pituitary gland, which acts on the adrenal glands to release CORT, and in turn, exerts a negative feedback effect on the pituitary gland

to regulate ACTH release. The resultant pulsatile dynamic is due to the time delay in the adrenal gland *de novo* synthesis of CORT, and in the negative feedback, regulation to inhibit ACTH release from the pituitary gland. Pulsatile ACTH release, and the subsequent pulsatile pattern of CORT, has been well established in the rat (111, 131, 133, 134) and humans (109). As shown in chapter 3, SAFit1 enhances the GC negative feedback in the anterior pituitary gland, and this would increase the rate of the negative feedback loop to the adrenal glands, as well as reduce the ACTH secretion, and result in shorter CORT pulses and less overall CORT. This is indeed observed following sub-chronic SAFit1 treatment. However, SAFit2 also enhances the negative feedback in the anterior pituitary gland, as shown in the CRH experiment (figure 3.9 and figure 3.10), but under basal conditions, does not affect the number of pulses, the pulse length, or the IPI (figure 4.3). The data in this thesis are not sufficient to explain the mechanisms underlying the effects of SAFit1 on the CORT pulsatile dynamics. However, mathematical modelling studies of the system that will take into account the activity of FKBP5 and its inhibition are on-going. Results from these mathematical modelling studies will certainly help elucidate the role of FKBP5 in regulating ultradian pulse dynamics and will allow predictions on how the system responds to perturbation on GR and FKBP5 signalling, in a region- and time-dependent manner.

7.4. Effects of SAFit2 and SAFit1 treatment on the HPA axis activity in female rats

The second aim was to determine the effects of acute and prolonged administration of SAFit2 and SAFit1 on basal and stress-induced CORT secretion in female adult rats, and investigate whether these effects differ from the effects seen in male rats and if so, to attempt to elucidate the mechanisms underlying these differences. This aim was driven by the well-known sexual dimorphism in the HPA axis activity in both humans and rats (reviewed in (231) – section 1.9). This HPA axis dimorphism is characterised by increased basal and stress-induced CORT concentrations in female rats (231). Whereas in humans the results are more varied, women show an increased stress-response to certain stressors including social interaction and rejection (262), and pharmacological stressors (260). Interestingly, one study showed that women have reduced GC-mediated negative feedback compared to men when exposed to high-intensity exercise as a stressor (264).

Hence, this difference in HPA axis regulation is characterised by different sensitivity to certain stressors in men and women. It is indeed suggested that dysfunctional GC-mediated negative feedback, and thus an inability to terminate a stress response adequately, is present in patients with certain mood disorders such as depression (71, 270, 438, 439). Although the molecular basis for the sex differences in HPA axis activity is not fully understood, studies in both humans and rodents suggest that females have decreased GC negative feedback. For example, the DEX-CRH test results in higher CORT concentrations in women (270, 307), and female rats have lower expression of GR in the PVN compared to males (239). It is plausible that similar mechanisms contribute to the higher prevalence of mental disorders in women (440).

In addition to the well-characterised sex differences in basal and stress-induced CORT secretion in rodents, it has been shown that female rats have increased serum CBG (up to 2.5 fold) compared to male rats (287), and CBG is positively regulated by oestrogen (441). This implies that free, biologically active CORT could be similar, or even lower in female rats. It is therefore plausible that in female rodents the higher levels of CBG compensate for higher CORT concentrations. This was investigated by measuring intrahippocampal ultradian rhythms of CORT in male and female rats in basal conditions and following stress (119, 442). Because CBG is not present in the brain this measured the free, active, CORT. The studies found similar concentrations, and similar circadian and ultradian pulses of hippocampal CORT in male and female rats (119, 442).

In this thesis, it was determined whether there were any effects of acute treatment with SAFit2 or SAFit1 on basal and stress-induced CORT. Unfortunately, due to limited SAFit1 availability and time constraints, it was not possible to characterise the effects of sub-chronic SAFit1 treatment in female rats. Furthermore, the expression of GR, MR, and FKBP5 in the hippocampus, hypothalamus, prefrontal cortex, and the anterior pituitary gland, in male and female control rats, in basal conditions both in the morning (9 AM) and in the evening (5 PM) was determined. The hypothesis was that, if the expression profile of those genes is different in males and females, this difference could contribute to any potential differences in the effects of SAFit2 or SAFit1 in male and female rats.

Acute SAFit2 administration in female rats did not affect the basal 24-hour CORT concentrations (figure 5.3), however, it decreased stress-induced CORT (figure 5.7 A). In

contrast, acute SAFit1 administration significantly decreased both basal (figure 5.5) and stress-induced (5.7 B) CORT concentrations in female rats. The decrease in basal CORT was evidenced by decreased mean CORT and CORT AUC. Gene expression analysis following the noise stress did not reveal any effect of the SAFit2 treatment on any of the genes quantified (figure 5.11 A, C, and figure 5.12 A, C). In contrast, acute SAFit1 treatment significantly increased FKBP5 mRNA in the anterior pituitary gland (figure 5.12 C).

Sub-chronic administration of SAFit2 in female rats significantly decreased basal CORT concentrations (figure 5.8), however, there was no difference in stress-induced CORT concentrations compared to vehicle-treated rats (figure 5.10). The reduction in basal CORT was due to significantly lower pulse height, pulse amplitude, and pulse area, and therefore lower mean, maximum and baseline CORT. The gene expression analysis following noise stress revealed an increase in PER1, a GR-inducible gene, in the anterior pituitary gland in female rats treated sub-chronically with SAFit2 (figure 5.13 D).

The results from the gene expression quantification in untreated male and female rats showed that the major differences were in the anterior pituitary gland, where the overall expression of GR, MR, and FKBP5 was significantly lower in the females compared to males (figure 5.14). Seale and colleagues compared the expression of GR mRNA in the PVN in sham male, sham female, castrated males, and ovariectomised female (239). The study found that sham females had lower PVN GR mRNA compared to sham males, and castrated males have decreased GR mRNA compared to sham males. Further, ovariectomised females had increased GR mRNA compared to sham females (239). Although they did not investigate potential changes in GR mRNA in the pituitary gland, the study did show that sex steroids can influence GR expression. In the studies performed in chapter 5 of this thesis, there was no difference in GR mRNA between males and females in the hypothalamus, however, this might be because the whole hypothalamus was used, rather than isolated PVN. Overall, the results are consistent with the present study, where females have lower expression of GR mRNA compared to males. The same group performed a second study where the castrated and ovariectomised male and female rats were treated with respective sex steroids (240). The results indicated that when castrated males are treated with testosterone the GR mRNA in the PVN returns to similar levels as in sham males (240). Similarly, ovariectomised female rats treated with E2 decreased the GR mRNA in the PVN to levels similar to those in sham female

rats (240). Overall, this suggests that testosterone induces GR expression, and oestrogen inhibits GR expression, presumably via the AR and the ER, respectively. Although it is not yet known how sex steroids can influence the expression of GR and although, to my knowledge, no ARE or ERE has been discovered on the GR promoter, further research in this area might reveal a mechanism. Besides, CRH-neurons located in the PVN express ER-β (443) and it is thought that oestradiol-mediated activation increases CRH expression (444).

Mechanisms may have evolved to compensate for the higher CORT concentrations observed in female rats and mice (231). This might include the increased CBG in female rats as mentioned above (287). On the other hand, lower expression of GR and FKBP5 mRNA in the anterior pituitary gland of female rats compared to male rats was observed in the studies in chapter 5 (figure 5.14), and lower GR mRNA expression in females in the anterior pituitary gland has been observed in other studies (239), as mentioned. A lower expression of GR in the PVN and anterior pituitary gland might contribute to a decreased GC-mediated negative feedback in female rats. As the FKBP5 mRNA expression is also lower in the anterior pituitary gland of the female rats in the present study, perhaps this is to mitigate the effects of reduced GR availability. Although beyond the scope of this thesis it would be interesting to further characterise the expression of FKBP51 and GR in the pituitary gland and brain under various physiological conditions.

7.4.1. Effects of SAFit2 and SAFit1 treatment on basal and stress-induced CORT secretion in female rats

As SAFit2 has the same chemical characteristics as SAFit1, except that it can cross the BBB, SAFit2 should also be active peripherally. Indeed, in male rats, acute SAFit2 treatment result in lower ACTH, and subsequent CORT secretion, thus indicating that SAFit2 is active in the pituitary gland. Hence, as acute SAFit1 treatment in females result in significantly lower CORT concentrations, one would expect SAFit2 to also cause lower basal CORT, as the SAFit2 is also present and should be active in the periphery, similar to SAFit1. Moreover, SAFit2 should be active centrally in the females if it is assumed that SAFit2 can cross the BBB in females. The sub-chronic SAFit2 experiment in females suggests that the peripheral effects of SAFit2 diminish this putative central decrease in GC negative feedback, however, the stress-

induced CORT is not affected. The mechanism behind this is not clear, however as discussed in section 5.4, it may involve the ER-α in the PVN. Perhaps the stress-mediated increase in CRH during the stress response further enhances this centrally mediated decrease in negative feedback, and the peripheral actions of SAFit2 are not sufficient to diminish this. Intra-cranial injection of SAFit2 in female rats could be performed to further elucidate this.

Another interesting observation in the female studies was that acute SAFit2 does not affect stress-induced CORT secretion, however, SAFit1 significantly reduces stress-induced CORT secretion. This suggests that the stress-induced rapid negative feedback can be enhanced by inhibition of FKPB51 in the pituitary gland. There are several mechanisms by which this might occur, as discussed above. Compared to the male SAFit1 experiments there was no effect of acute SAFit1 on stress-induced CORT, however, a decrease was seen following sub-chronic SAFit1 treatment. Perhaps stress-induced rapid negative feedback is regulated to a greater part in the pituitary gland in females and therefore lower CORT concentrations are observed after acute treatment with SAFit1. If the stress-induced CORT is regulated to a greater part centrally in males then that could explain why prolonged treatment of SAFit1, and hence a build-up of the SAFit1 to render a stronger effect, is required to see an effect on the stress-induced CORT. Furthermore, as shown in chapter five (figure 5.14), untreated females have a lower basal expression of both GR and FKBP5 mRNA in the pituitary gland, compared to control males. Thus, the same dose of SAFit1 could be more effective in females due to less FKBP51 available.

7.4.2. Sex differences in SAFit2 and SAFit1 effects

The results reported in this thesis highlight several sex differences in FKBP51-mediated regulation of GC negative feedback under both basal conditions and subsequent stress. Even though both males and females responded to the SAFit2 and SAFit1 treatments, and there were several similarities in the response, several important differences were observed. The first remarkable difference was the effect on stress-induced CORT following sub-chronic treatment. In males, the stress-induced CORT was significantly decreased, whereas in females there was no effect of the SAFit2 treatment on stress-induced CORT secretion. As discussed above, this could be due to SAFit2 interfering with the oestrogen-

meditated activation of ERα in the PVN (401). More stress-related sex differences became apparent following acute SAFit1 treatment. In females, this caused a significant decrease in stress-induced CORT (figure 5.7), whereas in males there was no effect (figure 3.6). In comparison, acute SAFit2 treatment caused a significant decrease in stress-induced CORT in males (figure 3.6) whereas there was no effect in females (figure 5.7). Thus, acute administration of SAFit2 was effective in reducing stress-induced CORT secretion in males only, whilst SAFit1 was effective in reducing stress-induced CORT secretion in females only. In males, the lack of a decrease in stress-induced CORT following SAFit1 treatment, compared to a reduction in stress-induced CORT following SAFit2 suggests a central regulation of the stress-mediated negative feedback. However, sub-chronic SAFit1 treatment caused a reduction in stress-induced CORT in male rats. This indicates that the rapid GC negative feedback following stress is mediated by both central and pituitary gland mechanisms in male rats.

Previous studies have revealed that micro-infusion of CORT into the PVN, 1 hour or immediately before a stressor, inhibits ACTH secretion (143, 445). This suggests that the decrease in ACTH is due to decreased CRH stimulation. The decrease in CRH could be because of direct inhibition of the CRH neurons which, in turn, inhibits CRH secretion, or due to an indirect effect, such as the induction of retrograde endocannabinoid signalling which in turn regulates CRH neuron synaptic transmission (357). Another study investigated the effects of administering CORT at different time points before the onset of a stressor (446). Thus, this allows for a comparison of the negative feedback effects when the stress coincides with CORT's rapid rising phase (30 seconds before stress), high plateau phase (15 minutes before stress), and restored basal phase (60 minutes before stress). Administration of CORT at all three time points result in significantly lower ACTH secretion following restraint stress, and this reduction in ACTH was fully present at 5 minutes post-stress (446). In comparison, CORT administration 5 minutes post-onset of the restraint stress did not affect ACTH secretion. These results indicate that CORT administration as close as 30 seconds before a stressor significantly reduces stress-induced CORT secretion, thus suggesting that these effects may occur by non-genomic CORT actions (446). Moreover, the same study demonstrated that the effects of fast feedback lasted at least 15 minutes once initiated. This is consistent with the duration of *ex vivo* GC fast negative feedback in PVN slices, but not in corticotrophs (136, 144,

191). Furthermore, it was found that CRH hnRNA at 30 minutes post-stress was significantly decreased in rats receiving the 30-second CORT pre-treatment (446). This suggests that CORT-mediated GR activation also has rapid negative feedback actions to reduce CRH gene expression. The same study showed that the CORT treatment 5 minutes post-stress did not affect the stress-mediated increase in CRH hnRNA expression, but suppressed POMC hnRNA expression at the same time point (446). This indicates that resistance in stress-induced fast feedback is at the level of the PVN.

Numerous studies have reported GC-mediated rapid negative feedback in the pituitary gland as well (136, 200, 208) and have proposed several possible mechanisms, including changes in the cells electrophysiological properties (200), alterations to the actin cytoskeleton (208) and ligand-dependent membrane association of the GR (136). Deng and colleagues showed that CORT mediated ACTH suppression occurred rapidly but was short-lasting (approximately 5 minutes). The sustained effects of negative feedback seen *in vivo* following CORT administration (446) indicate that the stress-induced rapid negative feedback may not be mediated solely by corticotrophs and further supports a central regulation of the stress-induced negative feedback. Furthermore, in the *in vivo* experiment where sustained negative feedback was seen, to activate the negative feedback response a dose of CORT that is equivalent to stress-induced plasma levels of CORT (~400 ng/ml) was used. In contrast, Deng and colleagues used CORT concentrations equivalent to physiological concentrations of CORT (136). Thus, these studies indicate a central regulation of the stress-induced CORT negative feedback.

As discussed above, in comparison to male rats, central FKBP51-inhibition in female rats seem to counteract the pituitary gland effects of FKBP51-inhibition. As stress-induced CORT was significantly suppressed following acute SAFit1 treatment, and if we assume that SAFit2 has the same effects as SAFit1 on pituitary gland negative feedback, this infers that SAFit2 treatment increases CRH release. The CRH-mediated ACTH release is then reduced from the enhanced negative feedback in the pituitary gland by SAFit2. The same mechanism would explain the lack of stress-induced CORT in sub-chronic SAFit2-treated rats. The stress-induced CORT decreased following sub-chronic SAFit1 treatment in male rats, and following acute SAFit1 treatment in female rats, if the SAFit1 does not have central effects? A stressor induces increased HPA axis activity by increasing neuronal activation in certain brain regions,

including the hippocampus, amygdala, and the PVN directly (21). This, in turn, initiates the release of CRH from the PVN and an increase in ACTH and CORT secretion follows. The increased CORT secretion activates negative feedback by the mechanisms explained above. Thus, in the SAFit2-treated male rats, FKBP51-inhibition in the brain should enhance GR activity and this central negative feedback should ultimately reduce CRH release, and therefore decrease ACTH release. In addition, SAFit2 also enhances the negative feedback at the level of the pituitary gland, which further reduces ACTH release from the pituitary gland.

In contrast, in the SAFit1-treated rats, there is no enhanced negative feedback in the brain, hence the CRH that reaches the pituitary gland will be the same as in the vehicle-treated rats. However, in the pituitary gland, the SAFit1 enhances GC negative feedback, thus should reduce ACTH and CORT release, although to a lesser extent than in the SAFit2-treated rats. This is consistent with the result from the sub-chronic experiments in males, where the reduction of stress-induced CORT was more pronounced in the SAFit2-treated rats than in the SAFit1-treated rats. This is also consistent with the acute SAFit2 and SAFit2 experiments in males, where a small decrease in post-stress CORT was observed in SAFit2-treated rats, however, no decrease was seen in the SAFit1-treated rats. Thus the effect of acute SAFit1 was not sufficient to decrease stress-induced CORT, however, the effects of SAFit1 were enhanced when given for a prolonged time in line with studies of acute versus sub-chronic GR antagonists (383, 437).

Another striking difference between the males and females was the effect of acute SAFit1 treatment on basal HPA axis activity. In females, there was a significant decrease in mean CORT secretion in SAFit1-treated rats (figure 5.6), while in males there was no effect of SAFit1 treatment on the 24-hour basal CORT concentrations (figure 3.5). As there is lower expression of FKBP51 in the anterior pituitary gland in the control female rats compared to control male rats (figure 5.16), it is plausible that SAFit1 has a stronger effect in female rats because the SAFit1 can inhibit more of the available FKBP51. Furthermore, the gene expression profiles in the sub-chronic SAFit2-treated males and females differed. In the males, there was an increase in GR-inducible genes GILZ and SGK1 in the hypothalamus (figure 4.7 C), while in the females there was an increase in the GR-inducible gene PER1 in the anterior pituitary gland (figure 5.13 D). This indicates increased GR transcriptional activity in the hypothalamus in the SAFit2-treated male rats, whereas increased GR transcriptional activity

occurred in the anterior pituitary gland in the SAFit2-treated female rats. This is consistent with the hypothesis of no enhanced central negative feedback following SAFit2 treatment in females. It is also consistent with an increased stress-induced negative feedback in the pituitary gland following SAFit2 or SAFit1 treatment in females, while in males the enhanced central negative feedback seems to play a bigger role. It should be noted that the increase in these GR-inducible genes is occurring despite the reduced CORT in the SAFit2- or SAFit1-treated rats, suggesting an even stronger effect on GR transcriptional activation.

In summary, there are sex differences in the regulation of FKBP51-mediated GR activation in regards to GC negative feedback in male and female rats, with a stronger effect on negative feedback in the pituitary gland in females. Furthermore, it is plausible that FKBP51 inhibition, via ERα, increases CRH release in SAFit2-treated females. This data highlights the complexity of HPA axis regulation and the difference between males and females. The reason why females and males have differentially regulated HPA axis responses is a pivotal question, yet to be fully understood. In humans, women are more susceptible to social rejection than are men (262) and perhaps this is because in our ancestors, women possibly relied on protection from other humans when breastfeeding their offspring, although this is only a hypothesis.

Studies have shown that the prevalence of any mental health disorder is higher in women than in men (440), and twice as many women are diagnosed with anxiety compared to men (447). Increased awareness of HPA axis sexual dimorphisms will help elucidate the underlying mechanisms of disorders with altered HPA axis activity and differences in men and women. Further research is needed to fully understand how the regulation of HPA axis activity differs in males and females. In addition, this will have profound clinical implications with the potential to develop a gender-specific treatment for not only psychiatric disorders but also for synthetic GC treatment. For example, if women have decreased GR-mediated negative feedback compared to men, can this be counteracted by administering a GR agonist? Or, if men have increased levels of FKBP5 mRNA (as seen in male rats – section 5.3.8), can this be targeted by administering an FKBP51-inhibitor? Although these are only speculations, the clinical implications of elucidating the HPA axis sexual dimorphism are many and the data presented in this thesis has provided a stepping stone for further research in this area.

7.5. Molecular mechanisms underlying the effects of SAFit2 and SAFit1.

The third aim was to investigate the molecular mechanism underlying the effects of SAFit2 and SAFit1 on HPA axis activity that were observed in basal and stress conditions. This aim was based on the hypothesis that FKBP51 inhibition increases nuclear GR, and hence could increase transcriptional active GR. This would, in turn, include GR-mediated activation of GC negative feedback ultimately to reduce circulating CORT concentrations. To test this hypothesis, animals were treated with SAFit2, SAFit1, or vehicle, and then injected with either CORT (0.3 mg/kg, s.c.) or saline. The results indicated no major effect of SAFit2 or SAFit1 on sub-cellular localisation or activation of GR or MR (figure 3.14 and 3.15). An interesting observation made was that in the hippocampus, membrane-associated MR was observed, while in the anterior pituitary gland no MR was present in the cell membrane fraction. Although the distribution of MR is not the focus of this thesis, this is a novel finding and would be worth looking into further.

Furthermore, to determine whether there was an induction of GR-inducible genes, and hence increased GR transcriptional activity, the gene expression of various GR-inducible genes was measured following noise stress in all the acute and sub-chronic SAFit2 and SAFit1 sampling experiments, as described above. In summary, there was an induction of GR-inducible genes in the hypothalamus of sub-chronic SAFit2 males (figure 4.7 C), and the anterior pituitary gland of sub-chronic SAFit2 females (figure 5.13 D). For the acute studies, an increase in FKBP5, which is also GR-inducible (325, 326), was observed in the anterior pituitary gland in SAFit1-treated female rats (figure 5.12 D) but not in the SAFit1-treated male rats (figure 3.16 D). Hence, an increase in GR transcriptionally activity is observed following SAFit2 treatment in the hypothalamus of male rats, and the anterior pituitary gland of female rats. An increase in GR transcriptionally activity is observed in female rats in the anterior pituitary gland following acute SAFit1 treatment. The time points for the gene expression quantification was 60 minutes following noise stress, suggesting that GR translocates and enhances gene expression before 60 minutes. It is plausible that the timing of the sub-cellular localisation experiment contributed to the lack of effects seen.

The fact that there is no induction of the GR-regulated genes in the hypothalamus of the SAFit2-treated female rats (figure 5.11 A, C, and figure 5.13 A, B) further supports the hypothesis of a central decrease in negative feedback. Moreover, the increase in PER1

expression in the anterior pituitary of the sub-chronically SAFit2-treated females (figure 5.13 D) indicates SAFit2 indeed causes enhanced negative feedback in the pituitary gland. Furthermore, there is a clear induction of the GR-inducible genes in the hypothalamus of the sub-chronically SAFit2-treated males, however, there is no effect in the pituitary gland, also consistent with the ABS studies and noise stress experiment. Unfortunately, the translocation study did not reveal the molecular mechanism behind these effects. The gene expression analysis suggests that the effects of SAFit2 are due, at least partly, to genomic actions. In contrast, the results suggest that the effects of SAFit1, at least in male rats, are due to non-genomic effects.

In conclusion, these data further support the evidence for a centrally regulated stress-induced CORT negative feedback. Moreover, it is plausible that the ultradian pulsatility is at least partly regulated by non-genomic GR activity, as there was no induction of GR-inducible genes in the anterior pituitary gland following SAFit2 or SAFit1 treatment.

7.6. Future studies

To further elucidate the effects of SAFit1 treatment in females, it would be interesting to perform sub-chronic SAFit1 treatment in female rats. It is plausible that the decrease in basal and stress-induced CORT would be further enhanced. Moreover, it would be interesting to investigate gene expression following noise stress in such an experiment. In the acute SAFit1-treated female rats an increase in the GR-inducible FKBP5 mRNA following noise stress was observed in the anterior pituitary gland, suggesting increased GR transcriptional activity. It would be interesting to investigate whether prolonged SAFit1 treatment in females would increase the expression of other GR-inducible genes. In addition, performing similar CRH and ACTH experiments to those in chapter 3 in female rats would help further elucidate the observed sex differences in the central and peripheral effects of SAFit2 and SAFit1 on GC negative feedback. Furthermore, using ChIP on brain tissue to further investigate the effect of SAFit2 and SAFit1 on GR-DNA interactions could be a better way of detecting alterations in transcriptional activity, rather than the translocation experiment I performed. Similarly, using ISH to detect mRNA changes in distinct regions would be useful, as this would include

anatomical representation of the potential changes in gene expression and could, for example, show differences in gene expression between subregions of the hypothalamus.

The molecular mechanisms underlying the effects of SAFit2 and SAFit1 are yet to be fully elucidated. The translocation study (section 3.3.7) did not reveal any effects on GR translocation or phosphorylation following SAFit2 or SAFit1. Perhaps performing the same study with a higher dose of CORT, and at different time points for tissue collection, would reveal potential differences. The gene expression quantification following the noise stress does indeed suggest an increase in GR transcriptional activity. Performing the western immunoblotting to examine intracellular receptor translocation following sub-chronic treatment of SAFit2 or SAFit1 could reveal a stronger effect of the putative enhanced GR translocation. Furthermore, a more comprehensive gene expression analysis at the diurnal peak, following SAFit2 and SAFit1, could help elucidate any potential basal differences in gene expression.

The potential for FKBP51-inhibitors as therapeutics for disorders with a dysregulated HPA axis activity, such as depression, is promising. In the present study, SAFit2 and SAFit1 were tested in healthy male and female rats and the next step would be to perform similar studies in rats with a disrupted HPA axis. For example, rats exposed to chronic mild stress (CMS) have been shown to comprise a translational model of depression as indicated by the rat depression-like behavioural phenotype (448). Thus, administering SAFit2 and SAFit1 in a rat model of depression will help determine how the FKBP51-inhibition will affect a dysregulated HPA axis. For such studies, it might be advantageous to use an alternative to the subcutaneous administration of the SAFit2 and SAFit1, as the infection risk with the subcutaneous cannula increases with prolonged use. Other studies using SAFit2 in mice have explored a gel formulation, with good results (350, 351, 423). The SAFit2 was combined with a vesicular phospholipid gel (VPG) as a carrier, and it was shown that this results in stable plasma and brain concentrations of SAFit2 (351, 423).

To investigate the potential for FKBP51 as therapeutics for disorders involving HPA axis dysregulation, it is important to establish first a rat model with a hyperactive HPA axis and overexpression of FKBP51. Naturally occurring FKBP51 overexpression is present in squirrel monkeys, as described in the introduction (322, 331, 332), and FKBP51 overexpression has been induced in the mouse (340), however, to my knowledge there is no

model for naturally occurring or induced overexpression of FKBP51 in the rat. Therefore, I decided to investigate the potential for two new rat models of dysregulated HPA axis activity, the Fischer rat, and induced overexpression of FKBP51.

7.7. Pilot studies of dysregulated HPA axis activity

In chapter 6 it was demonstrated that male Fischer rats have increased FKBP51 expression in the anterior pituitary gland, compared to the histocompatible Lewis rats (figure 6.8). This is an important discovery, as it implicates the Fischer rats as a potential model for studying dysregulated HPA axis activity. Further, increased FKBP51 expression in humans has been linked to depression (407) and hence the Fischer rat model could be translational to humans. Characterising the potential effects of SAFit2 and SAFit1 on Fischer and Lewis rats can provide further insight into the involvement of FKBP51 and GR in HPA axis regulation.

In chapter 6 the effects of FKBP5 overexpression in the PVT of the thalamus were also discussed. Very little is known regarding the role of the thalamus in the regulation of the HPA axis activity, and even less is known regarding the involvement of FKBP51 in this regulation. The results from the study indicate that increased GR activity in the PVT alters ultradian CORT pulsatility resulting in enhanced pulse frequency, and reduced stress-induced CORT. These findings will provide new insight to further research into the involvement of the thalamus in HPA axis regulation.

7.8. Limitations of studies

The main limitation of the studies presented in this thesis was the availability of SAFit2 and SAFit1, which were not commercially produced (when these experiments were conducted) and had to be synthesised on request. It would have been useful to perform similar studies with GR agonists, as although this would not elucidate the effect of FKBP51, it would give further insight in HPA axis regulation by GR. For example, one could administer GR agonists centrally and peripherally, peripherally only, or intra-cranially, and then perform ABS studies to evaluate the effects on basal and stress-induced CORT. Furthermore, it would have been interesting to perform micro-infusions of SAFit2 directly into the PVN and evaluate the

effects of these, although due to limited availability of the SAFit2, and time limitations this was not possible.

The translocation experiment had limitations in that the technique used was not highly sensitive in quantifying the expression of GR. The experiment was also limited to one time point of tissue harvesting following the CORT injection, and this was based on previous studies (359). The CORT concentrations following the CORT injection were lower in this experiment than previously published (359) and perhaps even though the pre-administration of SAFit2 or SAFit1 could alter the dynamic of the GR translocation the effects would have been evident had a higher CORT dose been given, or if more time points had been investigated. It should also be noted that it appears some of the adrenalectomies were only partial as CORT concentrations were detected in the vehicle-saline group. Chromatin immunoprecipitation can be used for investigating DNA binding activity (380) and would have been a suitable option for the translocation study.

The surgical techniques were well-established within the group however patency of the cannulas is a common issue in cannulated animals and could perhaps be further improved. This issue was also further complicated by the limited availability of the SAFit2 and SAFit1 as I could not cannulate 'spare' rats to use in case some lost the cannula patency. This was especially apparent during the sub-chronic studies where large amounts of the SAFit compounds were required. Because of the quantities required, spare rats could not be treated and used if any of the SAFit-treated rats lost the cannula patency.

The Fischer and Lewis study had limitations in that due to the animal numbers being low and it was not possible to perform statistical analysis on the PULSAR data. This was mainly due to difficulties with the cannulation surgery and the ABS. As the Fischer rats were smaller in size than Sprague-Dawley rats this made successful cannulation more difficult due to smaller jugular veins.

The overexpression study had limitations because it was the first time this AAV-mediated overexpression had been done in rats hence we had no reference point for volume, time for full expression, etc. In addition, this study was particularly complicated as the rats underwent two surgeries in a short amount of time. Although it is still unclear why we were

unsuccessful in targeting the PVN, this led to a discovery of a strong involvement of FKBP51-mediated thalamic input to regulate HPA axis activity.

As previously mentioned, the stress-induced gene expression data from the ABS experiments have limitations in that only one time-point was investigated, as all rats were killed 60 to 90 minutes following the noise stress. Although, previous studies have shown that some of the genes investigated are indeed significantly induced at 60 minutes post-stress (13, 355).

Rats that were implanted with jugular vein cannula were injected with pentobarbital before rapid decapitation (less than 30 seconds). In some experiments where rats were not cannulated, rats were killed quickly by isoflurane exposure (less than 15 seconds) before rapid decapitation. Work from our group and others has shown that this procedure does not induce an increase in ACTH or CORT (132, 355).

7.9. Clinical implications and perspective

A vast number of studies have focused on elucidating the molecular mechanisms underlying the dysregulation of HPA axis activity observed in various psychiatric disorders such as major depression, bipolar disorder, schizophrenia, and anxiety disorders (71, 137). It is suggested that hyperactivity of the HPA axis in these disorders results from a failure to terminate the cortisol response to stress, thus creating stress-hypersensitivity. This mechanism is thought to be as a result of a dysfunctional GR activity (71), rendering an inadequate GC negative feedback response (137).

Recent interest in the GR chaperone, FKBP51, was sparked when Binder and colleagues published data suggesting a strong link between polymorphism in the gene encoding FKBP51, and the response to antidepressants (334). This led to an intriguing hypothesis that FKBP51 is implicated in the dysregulation of GC negative feedback seen in patients with psychiatric disorders (137). Most studies hypothesise that depressed patients have increased FKBP51 levels (339, 407, 449) which causes GR resistance (450).

The results from the experiments in this thesis have shown that central and peripheral inhibition of the FKBP51 with SAFit2 in male and female rats for five consecutive days results

in decreased basal CORT concentrations in both sexes, and decreased stress-induced CORT in male rats. Administration of the peripheral-only FKBP51-inhibitor, SAFit1, for five consecutive days resulted in a change in ultradian CORT pulsatility. This is consistent with the hypothesis of a pituitary-adrenal gland feed-forward/feedback pulse generator. This work has taken important steps in further unravelling the complexity of HPA axis regulation and will be useful for future studies exploring the use of FKBP51-inhibitors as a treatment for psychiatric disorders with increased HPA axis activity.

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